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A61P 37/04Center, University of Latvia, Ratsupites Iela 1, LV-1067  
Riga (LV).

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(72) Inventors; and

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16 September 2002 (16.09.2002)(75) Inventors/Applicants (for US only): **BACHMANN, Martin, F.** [CH/CH]; Tachlisbrunnenstrasse 30, CH-8400 Winterthur (CH). **STORNI, Tazio** [CH/CH]; Via Aldesago 10, CH-6962 Viganello (CH).

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(71) Applicant (for all designated States except US): **CYTOS BIOTECHNOLOGY AG** [CH/CH]; Wagistrasse 25, CH-8952 Zürich-Schlieren (CH).

(71) Applicants and

(72) Inventors: **MAURER, Patrick** [DE/CH]; Richenbergstrasse 38, CH-8400 Winterthur (CH). **TISSOT, Alain** [CH/CH]; Brauerstrasse 78, CH-8004 Zürich (CH). **SCHWARZ, Katrin** [DE/CH]; Schulstrasse 15, CH-8952 Schlieren (CH). **MEIJERINK, Edwin** [NL/CH]; Rebergstrasse 50, CH-8049 Zurich (CH). **LIPOWSKY, Gerad** [DE/CH]; Im Eisernen Zeit 55, CH-8057 Zurich (CH). **PUMPENS, Paul** [LV/LV]; Biomedical Research and Study Center, University of Latvia, Ratsupites Iela 1, LV-1067 Riga (LV). **CIELENS, Indulis** [LV/LV]; Biomedical Research and Study Center, University of Latvia, Ratsupites Iela 1, LV-1067 Riga (LV). **RENHOFA, Regina** [LV/LV]; Biomedical Research and Study

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(54) Title: PACKAGING OF IMMUNOSTIMULATORY SUBSTANCES INTO VIRUS-LIKE PARTICLES: METHOD OF PREPARATION AND USE

(57) Abstract: The invention relates to the finding that virus like particles (VLPs) can be loaded with immunostimulatory substances, in particular with DNA oligonucleotides containing non-methylated C and G (CpGs). Such CpG- VLPs are dramatically more immunogenic than their CpG-free counterparts and induce enhanced B and T cell responses. The immune response against antigens optionally coupled, fused or attached otherwise to the VLPs is similarly enhanced as the immune response against the VLP itself. In addition, the T cell responses against both the VLPs and antigens are especially directed to the Th1 type. Antigens attached to CpG-loaded VLPs may therefore be ...~ ideal vaccines for prophylactic or therapeutic vaccination against allergies, tumors and other self-molecules and chronic viral diseases.

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## Packaging of Immunostimulatory Substances Into Virus-Like Particles: Method of Preparation and Use

### *Background of the Invention*

#### *Field of the Invention*

The present invention is related to the fields of vaccinology, immunology and medicine. The invention provides compositions and methods for enhancing immunological responses against virus-like particles (VLPs) or against antigens coupled, fused or attached otherwise to VLPs by packaging immunostimulatory substances, in particular immunostimulatory nucleic acids, and even more particular oligonucleotides containing at least one non-methylated CpG sequence, into the VLPs. The invention can be used to induce strong and sustained T cell responses particularly useful for the treatment of tumors and chronic viral diseases as well as allergies and other chronic diseases.

#### *Related Art*

The essence of the immune system is built on two separate foundation pillars: one is specific or adaptive immunity which is characterized by relatively slow response-kinetics and the ability to remember; the other is non-specific or innate immunity exhibiting rapid response-kinetics but lacking memory. Lymphocytes are the key players of the adaptive immune system. Each lymphocyte expresses antigen-receptors of unique specificity. Upon recognizing an antigen via the receptor, lymphocytes proliferate and develop effector function. Few lymphocytes exhibit specificity for a given antigen or pathogen, and massive proliferation is usually required before an effector response can be measured - hence, the slow kinetics of the adaptive immune system. Since a significant proportion of the expanded lymphocytes survive and may maintain some effector function following elimination of the antigen,



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the adaptive immune system reacts faster when encountering the antigen a second time. This is the basis of its ability to remember.

In contrast to the situation with lymphocytes, where specificity for a pathogen is confined to few cells that must expand to gain function, the cells and molecules of the innate immune system are usually present in massive numbers and recognize a limited number of invariant features associated with pathogens (Medzhitov, R. and Janeway, C.A., Jr., *Cell* 91:295-298 (1997)). Examples of such patterns include lipopolysaccharides (LPS), non-methylated CG-rich DNA (CpG) or double stranded RNA, which are specific for bacterial and viral infections, respectively.

Most research in immunology has focused on the adaptive immune system and only recently has the innate immune system entered the focus of interest. Historically, the adaptive and innate immune system were treated and analyzed as two separate entities that had little in common. Such was the disparity that few researchers wondered why antigens were much more immunogenic for the specific immune system when applied with adjuvants that stimulated innate immunity (Sotomayor, E. M., *et al.*, *Nat. Med.* 5:780 (1999); Diehl, L., *et al.*, *Nat. Med.* 5:774 (1999); Weigle, W. O., *Adv. Immunol.* 30:159 (1980)). However, the answer posed by this question is critical to the understanding of the immune system and for comprehending the balance between protective immunity and autoimmunity.

Rationalized manipulation of the innate immune system and in particular activation of APCs involved in T cell priming to deliberately induce a self-specific T cell response provides a means for T cell-based tumor-therapy. Accordingly, the focus of most current therapies is on the use of activated dendritic cells (DCs) as antigen-carriers for the induction of sustained T cell responses (Nestle *et al.*, *Nat. Med.* 4:328 (1998)). Similarly, in vivo activators of the innate immune system, such as CpGs or anti-CD40 antibodies, are applied together with tumor cells in order to enhance their immunogenicity (Sotomayor, E. M., *et al.*, *Nat. Med.* 5:780 (1999); Diehl, L., *et al.*, *Nat. Med.* 5:774 (1999)).

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Generalized activation of APCs by factors that stimulate innate immunity may often be the cause for triggering self-specific lymphocytes and autoimmunity. Activation may result in enhanced expression of costimulatory molecules or cytokines such as IL-12 or IFN $\alpha$ . This view is compatible with the observation that administration of LPS together with thyroid extracts is able to overcome tolerance and trigger autoimmune thyroiditis (Weigle, W. O., *Adv. Immunol.* 30:159 (1980)). Moreover, in a transgenic mouse model, it was recently shown that administration of self-peptide alone failed to cause auto-immunity unless APCs were activated by a separate pathway (Garza, K. M., *et al.*, *J. Exp. Med.* 191:2021 (2000)). The link between innate immunity and autoimmune disease is further underscored by the observation that LPS, viral infections or generalized activation of APCs delays or prevents the establishment of peripheral tolerance (Vella, A. T., *et al.*, *Immunity* 2:261 (1995); Ehl, S., *et al.*, *J. Exp. Med.* 187:763 (1998); Maxwell, J. R., *et al.*, *J. Immunol.* 162:2024 (1999)). In this way, innate immunity not only enhances the activation of self-specific lymphocytes but also inhibits their subsequent elimination. These findings may extend to tumor biology and the control of chronic viral diseases.

Induction of cytotoxic T lymphocyte (CTL) responses after immunization with minor histocompatibility antigens, such as the HY-antigen, requires the presence of T helper cells (Th cells) (Husmann, L. A., and M. J. Bevan, *Ann. NY. Acad. Sci.* 532:158 (1988); Guerder, S., and P. Matzinger, *J. Exp. Med.* 176:553 (1992)). CTL-responses induced by cross-priming, *i.e.* by priming with exogenous antigens that reached the class I pathway, have also been shown to require the presence of Th cells (Bennett, S. R. M., *et al.*, *J. Exp. Med.* 186:65 (1997)). These observations have important consequences for tumor therapy where T help may be critical for the induction of protective CTL responses by tumor cells (Ossendorp, F., *et al.*, *J. Exp. Med.* 187:693 (1998)).

An important effector molecule on activated Th cells is the CD40-ligand (CD40L) interacting with CD40 on B cells, macrophages and dendritic

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cells (DCs) (Foy, T.M., *et al.*, *Annu. Rev. Immunol.* 14:591 (1996)). Triggering of CD40 on B cells is essential for isotype switching and the generation of B cell memory (Foy, T. M., *et al.*, *Ann. Rev. Immunol.* 14:591 (1996)). More recently, it was shown that stimulation of CD40 on macrophages and DCs leads to their activation and maturation (Cella, M., *et al.*, *Curr. Opin. Immunol.* 9:10 (1997); Banchereau, J., and R. M. Steinman *Nature* 392:245 (1998)). Specifically, DCs upregulate costimulatory molecules and produce cytokines such as IL-12 upon activation. Interestingly, this CD40L-mediated maturation of DCs seems to be responsible for the helper effect on CTL responses. In fact, it has recently been shown that CD40-triggering by Th cells renders DCs able to initiate a CTL-response (Ridge, J. P., *et al.*, *Nature* 393:474 (1998); Bennett, S. R. M., *et al.*, *Nature* 393:478 (1998); Schoenenberger, S. P., *et al.*, *Nature* 393:480 (1998)). This is consistent with the earlier observation that Th cells have to recognize their ligands on the same APC as the CTLs, indicating that a cognate interaction is required (Bennett, S. R. M., *et al.*, *J. Exp. Med.* 186:65 (1997)). Thus CD40L-mediated stimulation by Th cells leads to the activation of DCs, which subsequently are able to prime CTL-responses. In the human, type I interferons, in particular interferon  $\alpha$  and  $\beta$  may be equally important as IL-12.

In contrast to these Th-dependent CTL responses, viruses are often able to induce protective CTL-responses in the absence of T help (for review, see (Bachmann, M. F., *et al.*, *J. Immunol.* 161:5791 (1998)). Specifically, lymphocytic choriomeningitis virus (LCMV) (Leist, T. P., *et al.*, *J. Immunol.* 138:2278 (1987); Ahmed, R., *et al.*, *J. Virol.* 62:2102 (1988); Battegay, M., *et al.*, *Cell Immunol.* 167:115 (1996); Borrow, P., *et al.*, *J. Exp. Med.* 183:2129 (1996); Whitmire, J. K., *et al.*, *J. Virol.* 70:8375 (1996)), vesicular stomatitis virus (VSV) (Kündig, T. M., *et al.*, *Immunity* 5:41 (1996)), influenza virus (Tripp, R. A., *et al.*, *J. Immunol.* 155:2955 (1995)), vaccinia virus (Leist, T. P., *et al.*, *Scand. J. Immunol.* 30:679 (1989)) and ectromelia virus (Buller, R., *et al.*, *Nature* 328:77 (1987)) were able to prime CTL-responses in mice depleted of CD4<sup>+</sup> T cells or deficient for the expression of class II or CD40. The

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mechanism for this Th cell independent CTL-priming by viruses is presently not understood. Moreover, most viruses do not stimulate completely Th cell independent CTL-responses, but virus-specific CTL-activity is reduced in Th-cell deficient mice. Thus, Th cells may enhance anti-viral CTL-responses but the mechanism of this help is not fully understood yet. DCs have recently been shown to present influenza derived antigens by cross-priming (Albert, M. L., *et al.*, *J. Exp. Med.* 188:1359 (1998); Albert, M. L., *et al.*, *Nature* 392:86 (1998)). It is therefore possible that, similarly as shown for minor histocompatibility antigens and tumor antigens (Ridge, J. P., *et al.*, *Nature* 393:474 (1998); Bennett, S. R. M., *et al.*, *Nature* 393:478 (1998); Schoenenberger, S. P., *et al.*, *Nature* 393:480 (1998)), Th cells may assist induction of CTLs via CD40 triggering on DCs. Thus, stimulation of CD40 using CD40L or anti-CD40 antibodies may enhance CTL induction after stimulation with viruses or tumor cells.

However, although CD40L is an important activator of DCs, there seem to be additional molecules that can stimulate maturation and activation of DCs during immune responses. In fact, CD40 is not measurably involved in the induction of CTLs specific for LCMV or VSV (Ruedl, C., *et al.*, *J. Exp. Med.* 189:1875 (1999)). Thus, although VSV-specific CTL responses are partly dependent upon the presence of CD4<sup>+</sup>T cells (Kündig, T. M., *et al.*, *Immunity* 5:41 (1996)), this helper effect is not mediated by CD40L. Candidates for effector molecules triggering maturation of DCs during immune responses include Trance and TNF $\alpha$  (Bachmann, M. F., *et al.*, *J. Exp. Med.* 189:1025 (1999); Sallusto, F., and A. Lanzavecchia, *J Exp Med* 179:1109 (1994)).

It is well established that the administration of purified proteins alone is usually not sufficient to elicit a strong immune response; isolated antigen generally must be given together with helper substances called adjuvants. Within these adjuvants, the administered antigen is protected against rapid degradation, and the adjuvant provides an extended release of a low level of antigen.

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Unlike isolated proteins, viruses induce prompt and efficient immune responses in the absence of any adjuvants both with and without T-cell help (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Although viruses often consist of few proteins, they are able to trigger much stronger immune responses than their isolated components. For B cell responses, it is known that one crucial factor for the immunogenicity of viruses is the repetitiveness and order of surface epitopes. Many viruses exhibit a quasi-crystalline surface that displays a regular array of epitopes which efficiently crosslinks epitope-specific immunoglobulins on B cells (Bachmann & Zinkernagel, *Immunol. Today* 17:553-558 (1996)). This crosslinking of surface immunoglobulins on B cells is a strong activation signal that directly induces cell-cycle progression and the production of IgM antibodies. Further, such triggered B cells are able to activate T helper cells, which in turn induce a switch from IgM to IgG antibody production in B cells and the generation of long-lived B cell memory - the goal of any vaccination (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Viral structure is even linked to the generation of anti-antibodies in autoimmune disease and as a part of the natural response to pathogens (*see Fehr, T., et al., J. Exp. Med.* 185:1785-1792 (1997)). Thus, antigens on viral particles that are organized in an ordered and repetitive array are highly immunogenic since they can directly activate B cells.

In addition to strong B cell responses, viral particles are also able to induce the generation of a cytotoxic T cell response, another crucial arm of the immune system. These cytotoxic T cells are particularly important for the elimination of non-cytopathic viruses such as HIV or Hepatitis B virus and for the eradication of tumors. Cytotoxic T cells do not recognize native antigens but rather recognize their degradation products in association with MHC class I molecules (Townsend & Bodmer, *Ann. Rev. Immunol.* 7:601-624 (1989)). Macrophages and dendritic cells are able to take up and process exogenous viral particles (but not their soluble, isolated components) and present the generated degradation product to cytotoxic T cells, leading to their activation

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and proliferation (Kovacsovics-Bankowski *et al.*, *Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993); Bachmann *et al.*, *Eur. J. Immunol.* 26:2595-2600 (1996)).

Viral particles as antigens exhibit two advantages over their isolated components: (1) due to their highly repetitive surface structure, they are able to directly activate B cells, leading to high antibody titers and long-lasting B cell memory; and (2) viral particles, but not soluble proteins, have the potential to induce a cytotoxic T cell response, even if the viruses are non-infectious and adjuvants are absent.

Several new vaccine strategies exploit the inherent immunogenicity of viruses. Some of these approaches focus on the particulate nature of the virus particle; for example *see* Harding, C.V. and Song, R., (*J. Immunology* 153:4925 (1994)), which discloses a vaccine consisting of latex beads and antigen; Kovacsovics-Bankowski, M., *et al.* (*Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993)), which discloses a vaccine consisting of iron oxide beads and antigen; U.S. Patent No. 5,334,394 to Kossovsky, N., *et al.*, which discloses core particles coated with antigen; U.S. Patent No. 5,871,747, which discloses synthetic polymer particles carrying on the surface one or more proteins covalently bonded thereto; and a core particle with a non-covalently bound coating, which at least partially covers the surface of said core particle, and at least one biologically active agent in contact with said coated core particle (*see, e.g.*, WO 94/15585).

In a further development, virus-like particles (VLPs) are being exploited in the area of vaccine production because of both their structural properties and their non-infectious nature. VLPs are supermolecular structures built in a symmetric manner from many protein molecules of one or more types. They lack the viral genome and, therefore, are noninfectious. VLPs can often be produced in large quantities by heterologous expression and can be easily be purified.

In addition, DNA rich in non-methylated CG motifs (CpG), as present in bacteria and most non-vertebrates, exhibits a potent stimulatory activity on

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B cells, dendritic cells and other APC's *in vitro* as well as *in vivo*. Although bacterial DNA is immunostimulatory across many vertebrate species, the individual CpG motifs may differ. In fact, CpG motifs that stimulate mouse immune cells may not necessarily stimulate human immune cells and vice versa.

Although DNA oligomers rich in CpG motifs can exhibit immunostimulatory capacity, their efficiency is often limited, since they are unstable *in vitro* and *in vivo*. Thus, they exhibit unfavorable pharmacokinetics. In order to render CpG-oligonucleotides more potent, it is therefore usually necessary to stabilize them by introducing phosphorothioate modifications of the phosphate backbone.

A second limitation for the use of CpG-oligonucleotides to stimulate immune responses is their lack of specificity, since all APC's and B cells in contact with CpG-oligonucleotides become stimulated. Thus, the efficiency and specificity of CpG-oligonucleotides may be improved by stabilizing them or packaging them in a way that restricts cellular activation to those cells that also present the relevant antigen.

In addition, immunostimulatory CpG-oligodeoxynucleotides induce strong side effects by causing extramedullary hemopoiesis accompanied by splenomegaly and lymphadenopathy in mice (Sparwasser et al., J. Immunol. (1999), 162:2368-74 and Example 18).

VLPs have been shown to be efficiently presented on MHC class I molecules as they, presumably after uptake by macropinocytosis, are efficiently processed and crossprimed onto MHC class I. The mechanism of crosspriming is not clear to date, but TAP-dependent and TAP-independent pathways have been proposed.

There have been remarkable advances made in vaccination strategies recently, yet there remains a need for improvement on existing strategies. In particular, there remains a need in the art for the development of new and improved vaccines that promote a strong CTL immune response and anti-

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pathogenic protection as efficiently as natural pathogens in the absence of generalized activation of APCs and other cells.

### *Summary of the Invention*

This invention is based on the surprising finding that immunostimulatory substances such as DNA oligonucleotides can be packaged into VLPs which renders them more immunogenic. Unexpectedly, the nucleic acids and oligonucleotides, respectively, present in VLPs can be replaced specifically by the immunostimulatory substances and DNA-oligonucleotides containing CpG motifs, respectively. Surprisingly, these packaged immunostimulatory substances, in particular immunostimulatory nucleic acids such as unmethylated CpG-containing oligonucleotides retained their immunostimulatory capacity without widespread activation of the innate immune system. The compositions comprising VLP's and the immunostimulatory substances in accordance with the present invention, and in particular the CpG-VLPs are dramatically more immunogenic than their CpG-free counterparts and induce enhanced B and T cell responses. The immune response against antigens optionally coupled, fused or attached otherwise to the VLPs is similarly enhanced as the immune response against the VLP itself. In addition, the T cell responses against both the VLPs and antigens are especially directed to the Th1 type. Antigens attached to CpG-loaded VLPs may therefore be ideal vaccines for prophylactic or therapeutic vaccination against allergies, tumors and other self-molecules and chronic viral diseases.

In a first embodiment, the invention provides a composition for enhancing an immune response in an animal comprising a virus-like particle and an immunostimulatory substance, preferably an immunostimulatory nucleic acid, an even more preferably an unmethylated CpG-containing oligonucleotide, where the substance, nucleic acid or oligonucleotide is coupled, fused, or otherwise attached to or enclosed by, *i.e.*, bound, to the



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virus-like particle. In another embodiment, the composition further comprises an antigen bound to the virus-like particle.

In a preferred embodiment of the invention, the immunostimulatory nucleic acids, in particular the unmethylated CpG-containing oligonucleotides are stabilized by phosphorothioate modifications of the phosphate backbone. In another preferred embodiment, the immunostimulatory nucleic acids, in particular the unmethylated CpG-containing oligonucleotides are packaged into the VLPs by digestion of RNA within the VLPs and simultaneous addition of the DNA oligonucleotides containing CpGs of choice. In an equally preferred embodiment, the VLPs can be disassembled before they are reassembled in the presence of CpGs.

In a further preferred embodiment, the immunostimulatory nucleic acids do not contain CpG motifs but nevertheless exhibit immunostimulatory activities. Such nucleic acids are described in WO 01/22972. All sequences described therein are hereby incorporated by way of reference.

In a further preferred embodiment, the virus-like particle is a recombinant virus-like particle. Also preferred, the virus-like particle is free of a lipoprotein envelope. Preferably, the recombinant virus-like particle comprises, or alternatively consists of, recombinant proteins of Hepatitis B virus, BK virus or other human Polyoma virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth-Disease virus, Retrovirus, Norwalk virus or human Papilloma virus, RNA-phages, Q $\beta$ -phage, GA-phage, fr-phage and Ty. In a specific embodiment, the virus-like particle comprises, or alternatively consists of, one or more different Hepatitis B virus core (capsid) proteins (HBcAgs).

In a further preferred embodiment, the virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage. Preferred RNA-phages are Q $\beta$ -phage, AP 205-phage, GA-phage, fr-phage

In another embodiment, the antigen is a recombinant antigen. In yet another embodiment, the antigen can be selected from the group consisting of: (1) a polypeptide suited to induce an immune response against cancer cells; (2)

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a polypeptide suited to induce an immune response against infectious diseases; (3) a polypeptide suited to induce an immune response against allergens; (4) a polypeptide suited to induce an improved response against self-antigens; and (5) a polypeptide suited to induce an immune response in farm animals or pets.

In yet another embodiment, the antigen can be selected from the group consisting of: (1) an organic molecule suited to induce an immune response against cancer cells; (2) an organic molecule suited to induce an immune response against infectious diseases; (3) an organic molecule suited to induce an immune response against allergens; (4) an organic molecule suited to induce an improved response against self-antigens; (5) an organic molecule suited to induce an immune response in farm animals or pets; and (6) an organic molecule suited to induce a response against a drug, a hormone or a toxic compound.

In a particular embodiment, the antigen comprises, or alternatively consists of, a cytotoxic T cell epitope. In a related embodiment, the virus-like particle comprises the Hepatitis B virus core protein and the cytotoxic T cell epitope is fused to the C-terminus of said Hepatitis B virus core protein. In one embodiment, they are fused by a leucine linking sequence.

In another aspect of the invention, there is provided a method of enhancing an immune response in a human or other animal species comprising introducing into the animal a composition comprising a virus-like particle and immunostimulatory substance, preferably an immunostimulatory nucleic acid, an even more preferably an unmethylated CpG-containing oligonucleotide where the substance, preferably the nucleic acid, and even more preferably the oligonucleotide is bound (*i.e.* coupled, attached or enclosed) to the virus-like particle. In a further embodiment, the composition further comprises an antigen bound to the virus-like particle.

In yet another embodiment of the invention, the composition is introduced into an animal subcutaneously, intramuscularly, intranasally, intradermally, intravenously or directly into a lymph node. In an equally

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preferred embodiment, the immune enhancing composition is applied locally, near a tumor or local viral reservoir against which one would like to vaccinate.

In a preferred aspect of the invention, the immune response is a T cell response, and the T cell response against the antigen is enhanced. In a specific embodiment, the T cell response is a cytotoxic T cell response, and the cytotoxic T cell response against the antigen is enhanced.

The present invention also relates to a vaccine comprising an immunologically effective amount of the immune enhancing composition of the present invention together with a pharmaceutically acceptable diluent, carrier or excipient. In a preferred embodiment, the vaccine further comprises at least one adjuvant, such as incomplete Freund's adjuvant. The invention also provides a method of immunizing and/or treating an animal comprising administering to the animal an immunologically effective amount of the disclosed vaccine.

In a preferred embodiment of the invention, the immunostimulatory substance-containing VLPs, preferably the immunostimulatory nucleic acid-containing VLP's, an even more preferably the unmethylated CpG-containing oligonucleotide VLPs are used for vaccination of animals or humans against the VLP itself or against antigens coupled, fused or attached otherwise to the VLP. The modified VLPs can be used to vaccinate against tumors, viral diseases, self-molecules and self antigens, respectively, or non-peptidic small molecules, for example. The vaccination can be for prophylactic or therapeutic purposes, or both. Also, the modified VLPs can be used to vaccinate against allergies in order to induce immune-deviation.

In the majority of cases, the desired immune response will be directed against antigens coupled, fused or attached otherwise to the immunostimulatory substance-containing VLPs, preferably the immunostimulatory nucleic acid-containing VLP's, an even more preferably the unmethylated CpG-containing oligonucleotide VLPs. The antigens can be peptides, proteins, domains, carbohydrates or small molecules such as, for example, steroid hormones or drugs, such as nicotine. Under some conditions,

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the desired immune response can be directed against the VLP itself. This latter application will be used in cases where the VLP originates from a virus against which one would like to vaccinate.

The route of injection is preferably subcutaneous or intramuscular, but it would also be possible to apply the CpG-containing VLPs intradermally, intranasally, intravenously or directly into the lymph node. In an equally preferred embodiment, the CpG-containing antigen-coupled or free VLPs are applied locally, near a tumor or local viral reservoir against which one would like to vaccinate.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

### ***Brief Description Of The Drawings/Figures***

Figure 1 shows the DNA sequence of the CpG-oligonucleotide (A) and the DNA sequence of the peptide p33-containing VLP derived from hepatitis B core (B). The nonameric p33 epitope is genetically fused to the C-terminus of the hepatitis B core protein at position 185 via a three leucine linking sequence.

Figure 2 shows the structure of the p33-VLPs as assessed by electron microscopy (A) and SDS PAGE (B). Recombinantly produced wild-type VLPs (composed of HBcAg [aa 1-185] monomers) and p33-VLPs were loaded onto a Sephacryl S-400 gel filtration column (Amersham Pharmacia Biotechnology AG) for purification. Pooled fractions were loaded onto a Hydroxyapatite column. Flow through (which contains purified HBc capsids) was collected and loaded onto a reducing SDS-PAGE gel for monomer molecular weight analysis (B).

Figure 3 shows p33-VLPs in a native agarose gel electrophoresis (1% agarose) after control incubation or after digestion with RNase A upon staining with ethidium bromide (A) or Coomassie blue (B) in order to assess

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for the presence of RNA or protein. Recombinantly produced p33-VLPs were diluted at a final concentration of 0.5 ug/ul protein in PBS buffer and incubated in the absence (lane 1) or presence (lane 2) of RNase A (100 ug/ml) (Sigma, Division of Fluka AG, Switzerland) for 2 h at 37°C. The samples were subsequently complemented with 6-fold concentrated DNA-loading buffer (MBS Fermentas GmbH, Heidelberg, Germany) and run for 30 min at 100 volts in a 1% native agarose gel. The Gene Ruler marker (MBS Fermentas GmbH, Heidelberg, Germany) was used as reference for p33-VLPs migration velocity (lane M). Arrows are indicating the presence of RNA packaged in p33-VLPs (A) or p33-VLP capsids themselves (B). Identical results were obtained in 3 independent experiments.

Figure 4 shows p33-VLPs in a native agarose gel electrophoresis (1% agarose) after control incubation or after digestion with RNase A in the presence of buffer only or CpG-containing DNA-oligonucleotides upon staining with ethidium bromide (A) or Coomassie blue (B) in order to assess for the presence of RNA/DNA or protein. Recombinant p33-VLPs were diluted at a final concentration of 0.5 ug/ul protein in PBS buffer and incubated in the absence (lane 1) or presence (lane 2 and 3) of RNase A (100 ug/ml) (Sigma, Division of Fluka AG, Switzerland) for 2 h at 37°C. 5 nmol CpG-oligonucleotides (containing phosphorothioate modification of the backbone) were added to sample 3 before RNase A digestion. The Gene Ruler marker (MBS Fermentas GmbH, Heidelberg, Germany) was used as reference for p33-VLPs migration velocity (lane M). Arrows are indicating the presence of RNA or CpG-oligonucleotides in p33-VLPs (A) or p33-VLPs capsids themselves (B). Identical results were obtained when CpG oligonucleotides with phosphodiester bonds were used for co-incubation of VLPs with RNase A.

Figure 5 shows p33-VLPs in a native agarose gel electrophoresis (1% agarose) before and after digestion with RNase A in the presence of CpG-containing DNA-oligonucleotides and subsequent dialysis (for the elimination of VLP-unbound CpG DNA) upon staining with ethidium bromide (A) or

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Coomassie blue (B) in order to assess for the presence of DNA or protein. Recombinant p33-VLPs were diluted at a final concentration of 0.5 ug/ul protein in PBS buffer and incubated in absence (lane 1) or in presence (lanes 2 to 5) of RNase A (100 ug/ml) (Sigma, Division of Fluka AG, Switzerland) for 2 h at 37°C. 50 nmol CpG-oligonucleotides (containing phosphorothioate modification of the phosphate backbone: lanes 2 and 3, containing phosphodiester bonds: lanes 4 and 5) were added to VLPs before RNase A digestion. Treated samples were extensively dialysed for 24 hours against PBS (4500-fold dilution) with a 300 kDa MWCO dialysis membrane (Spectrum Medical Industries Inc., Houston, USA) to eliminate the in excess DNA (lanes 3 and 5). The Gene Ruler marker (MBS Fermentas GmbH, Heidelberg, Germany) was used as reference for p33-VLPs migration velocity (lane M). Arrows are indicating the presence of RNA or CpG-oligonucleotides in p33-VLPs (A) or p33-VLP capsids themselves (B).

Figure 6 shows p33-VLPs in a native agarose gel electrophoresis (1% agarose) after control incubation or after digestion with RNase A where CpG-containing oligonucleotides were added only after completing the RNA digestion upon staining with ethidium bromide (A) or Coomassie blue (B) in order to assess for the presence of RNA/DNA or protein. Recombinant p33-VLPs were diluted at a final concentration of 0.5 ug/ul protein in PBS buffer and incubated in the absence (lane 1) or presence (lane 2 and 3) of RNase A (100 ug/ml) (Sigma, Division of Fluka AG, Switzerland) for 2 h at 37°C. 5 nmol CpG-oligonucleotides (containing phosphorothioate modification of the phosphate backbone) were added to sample 3 only after the RNase A digestion. The Gene Ruler marker (MBS Fermentas GmbH, Heidelberg, Germany) was used as reference for p33-VLPs migration velocity (lane M). Arrows are indicating the presence of RNA or CpG-oligonucleotides in p33-VLPs (A) or p33-VLP capsids themselves (B). Identical results were obtained when CpG oligonucleotides with phosphodiester bonds were used for reassembly of VLPs.

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Figure 7 shows that p33-VLPs packaged with CpG-oligonucleotides (containing phosphorothioate modification of the phosphate backbone), are effective at inducing viral protection. Mice were subcutaneously primed with 100 µg p33-VLP alone, mixed with 20 nmol CpG-oligonucleotide (p33-VLP+CpG) or p33-VLP packaged with CpG-oligonucleotide after dialysis of free CpG-oligonucleotide (p33-VLP/CpG). Untreated naïve mice served as negative control. Twenty-one days later, mice were challenged with LCMV (200 pfu, intravenously) and viral titers were assessed in the spleens 5 days later as described in Bachmann, M. F., "Evaluation of lymphocytic choriomeningitis virus-specific cytotoxic T cell responses," in *Immunology Methods Manual*, Lefkowitz, I., ed., Academic Press Ltd., New York, NY (1997) p. 1921.

Figure 8 shows that p33-VLPs packaged with CpG-oligonucleotide (containing phosphodiester bonds) are effective at inducing viral protection. Mice were subcutaneously primed with 100 µg p33-VLP alone, mixed with 20 nmol CpG-oligonucleotides (p33-VLP+CpG) or p33-VLPs packaged with CpG-oligonucleotides after dialysis of free CpG-oligonucleotides (p33-VLP/CpG). Untreated naïve mice served as negative control. Twenty-one days later, mice were challenged with LCMV (200 pfu, intravenously) and viral titers were assessed in the spleens 5 days later as described in Bachmann, M. F., "Evaluation of lymphocytic choriomeningitis virus-specific cytotoxic T cell responses," in *Immunology Methods Manual*, Lefkowitz, I., ed., Academic Press Ltd., New York, NY (1997) p. 1921.

Figure 9 shows that mice treated with CpG-oligonucleotides alone are not protected from viral infection. Mice were subcutaneously primed with 20 nmol CpG-oligonucleotides (CpG), or left untreated as negative control (naïve). Twenty-one days later, mice were challenged with LCMV (200 pfu, intravenously) and viral titers were assessed in the spleens 5 days later as described in Bachmann, M. F., "Evaluation of lymphocytic choriomeningitis virus-specific cytotoxic T cell responses," in *Immunology Methods Manual*, Lefkowitz, I., ed., Academic Press Ltd., New York, NY (1997) p. 1921.

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Figure 10 shows the amino acid sequence of the BKV (AS) VP1 protein (GI:332779). This sequence was expressed in yeast to produce BKV capsids (Sasnauskas K. *et al.*, *J. Biol Chem* 380(3):381 (1999); K. *et al.*, *Generation of recombinant virus-like particles of different polyomaviruses in yeast*. 3<sup>rd</sup> International Workshop "Virus-like particles as vaccines" Berlin, (2001)).

Figure 11 shows the DNA sequence of the 246 bp double stranded DNA fragment used for packaging and stabilization of BKV VLPs.

Figure 12 shows BKV VLPs (15 µg) in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with fluorescent phosphorothioate (pt) CpG-oligonucleotides. UV excitation leads to detection of DNA in an ethidium bromide stained gel (A) and to fluorescence of CpG-FAM oligomers in the absence of ethidium bromide (B). Lane 1: BKV VLPs untreated; lane 2: BKV VLPs RNase A treated; lane 3: BKV VLPs RNase A treated with CpG(pt)-FAM; lane 4: BKV VLPs RNase A treated with CpG(pt)-FAM plus DNaseI treatment; lane M: Gene Ruler 1 kb DNA ladder (MBI Fermentas GmbH, Heidelberg, Germany). Arrows are indicating the presence of RNA or CpG-FAM oligomers in BKV VLPs.

Figure 13 shows BKV VLPs (15 µg) in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with double stranded (ds) DNA (246 bp) upon staining with ethidium bromide (A) or Coomassie Blue (B). Lane 1: BKV VLPs untreated; lane 2: BKV VLPs RNase A treated; lane 3: BKV VLPs treated with RNase A and incubated with ds DNA; lane M: Gene Ruler 1 kb DNA ladder (MBI Fermentas GmbH, Heidelberg, Germany). Arrows indicate the presence of RNA or ds DNA in BKV VLPs.

Figure 14 shows BKV VLPs (15 µg) in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with CpG-oligonucleotides (with phosphate- or with phosphorothioate (pt) backbone) upon staining with ethidium bromide (A) or



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Coomassie Blue (B). Lane 1: BKV VLPs stock (PBS/50% glycerol); lane 2: BKV VLPs untreated (PBS buffer); lane 3: BKV VLPs RNase A treated; lane 4: BKV VLPs RNase A treated post- dialysis; lane 5: BKV VLPs RNase A treated with CpG-oligonucleotides; lane 6: BKV VLPs RNase A treated with CpG(pt)-oligomers; lane 7: BKV VLPs RNase A treated with CpG(pt)-oligomers post-dialysis; lane M: Gene Ruler 1 kb DNA ladder (MBI Fermentas GmbH, Heidelberg, Germany). Arrows indicate the presence of RNA or CpG-oligonucleotides in BKV VLPs.

Figure 15 shows mouse IgG1 and IgG2a OD50% antibody titers to BKV VLPs on day 14 after immunization with BKV VLPs and phosphorothioate (pt) CpG-oligonucleotides. Lane 1: RNase treated BKV VLPs; lane 2: RNase treated BKV VLPs in combination with 0.3 nmol CpG(pt)-oligomer; lane 3: RNase treated BKV VLPs in combination with 20 nmol CpG(pt)-oligomer; lane 4: RNase treated BKV VLPs containing 0.3 nmol CpG(pt)-oligomer.

Figure 16 shows p33-VLPs in a native agarose gel electrophoresis (1% agarose) after control incubation or after digestion with RNase A where linear double-stranded DNA (350 base pairs long) was added only after completing the RNA digestion upon staining with ethidium bromide (A) or Coomassie blue (B) in order to assess for the presence of RNA/DNA or protein. Recombinant p33-VLPs were diluted at a final concentration of 0.5 ug/ul protein in PBS buffer and incubated in the absence (lane 1) or presence (lanes 2, 3 and 4) of RNase A (100 ug/ml) (Sigma, Division of Fluka AG, Switzerland) for 2 h at 37°C. Linear double-stranded DNA of 350 bp in length was added to sample 3 and 4 only after the RNase A digestion to a final concentration of 100 ng/ml and incubated for 3 hours at 37 °C. Sample 4 was further digested with DNase I (50 IU/ml)(Sigma, Division of Fluka AG, Switzerland) for additional 3 hours at 37 °C. The Gene Ruler marker (MBS Fermentas GmbH, Heidelberg, Germany) was used as reference for p33-VLPs migration velocity (lane M). Arrows are indicating the presence of RNA/dsDNA free or enclosed in p33-VLPs (A) and p33-VLPs (B).

Figure 17 shows packaging of B-CpG into HBc33 VLPs.

Figure 18 shows packaging of NKCPG into HBc33 VLPs.

Figure 19 shows packaging of g10gacga-PO into HBc33 VLPs.

Figure 20 shows packaging of CyCpG-150 into HBc33 VLPs.

Figure 21 shows packaging of NKCPGpt into HBcP1A VLPs.

Figure 22 shows coupling of p33 to HBcAg VLPs.

Figure 23 shows packaging of B-CpGpt into HBx33 VLPs.

Figure 24 shows coupling of p33 to Q $\beta$  VLPs.

Figure 25 shows ionic strength and low protein concentration allow RNA hydrolysis by RNase A in Q $\beta$  VLPs.

Figure 26 shows ionic strength increases immunostimulatory nucleic acid packaging into Q $\beta$  VLPs.

Figure 27 shows packaging of B-CpGpt, g10gacga-PO and dsCyCpG into Qbx33 VLPs.

Figure 28 shows SDS-PAGE analysis of the fractions from the sucrose gradient centrifugation after Q $\beta$  VLP disassembly and reassembly in the presence of immunostimulatory nucleic acids.

Figure 29 shows electron micrographs of Q $\beta$  VLP after disassembly and reassembly in the presence of oligonucleotide (CpG)<sub>20</sub>OpA.

Figure 30 shows ouchterlony analysis (immunodiffusion) of the disassembled and reassembled Q $\beta$  VLP.

Figure 31 shows gelelectrophoretic analysis of disassembled and reassembled Q $\beta$  VLP.

Figure 32 shows electron micrographs of the disassembled and reassembled Q $\beta$  VLP with the oligonucleotide CyOpA.

Figure 33 shows electron micrographs of the purified disassembled and reassembled Q $\beta$  VLP with the different immunostimulatory nucleic acids.

Figure 34 shows SDS-PAGE analysis of the coupling of Q $\beta$  VLP reassembled with the oligodeoxynucleotide CyOpA to the p33GGC peptide.

Figure 35 shows packaged oligodeoxynucleotides after disassembly and reassembly of Q $\beta$  VLPs and subsequent coupling to p33 GGC peptide.

Figure 36 shows purification of disassembled Q $\beta$  coat protein by size exclusion chromatography.

Figure 37 shows purification of reassembled Q $\beta$  VLPs by size exclusion chromatography.

Figure 38 shows electron micrographs of Q $\beta$  VLPs that were reassembled in the presence of different oligodeoxynucleotides.

Figure 39 shows analysis of the disulfide-bond pattern in reassembled and purified Q $\beta$  capsids.

Figure 40 shows analysis of nucleic acid content of the reassembled Q $\beta$  VLPs by nuclease treatment and agarose gelelectrophoresis.

Figure 41 shows analysis of nucleic acid content of the reassembled Q $\beta$  VLPs by proteinase K treatment and polyacrylamide TBE/Urea gelelectrophoresis.

Figure 42 shows electron micrographs AP205 VLP disassembled and subsequently reassembled in the presence of CyCpG.

Figure 43 shows agarose gel-electrophoresis analysis of AP205 VLPs disassembled and reassembled in the presence of CyCpG.

Figure 44 shows electron micrograph of disassembled and reassembled AP205.

Figure 45 shows Agarose gel-electrophoresis analysis of AP205 VLPs disassembled and reassembled in the presence of CyCpG.

Figure 46 shows SDS-PAGE analysis of disassembled and reassembled AP205 VLPs.

Figure 47 shows SDS-PAGE analysis of the peptide coupling to disassembled and reassembled AP205 VLPs.

Figure 48 shows free immunostimulatory nucleic acids but not immunostimulatory nucleic acids packaged in VLPs induce splenomegaly.

Figure 49 shows different immunostimulatory nucleic acids packaged in VLP fused to antigen result in a potent antigen-specific CTL response and virus protection.

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Figure 50 shows the immunostimulatory nucleic acid g10gacga-PS packaged in VLP fused to antigen result in a potent antigen-specific CTL response and virus protection.

Figure 51 shows immunostimulatory nucleic acids packaged in HBcAg and Q $\beta$  VLPs result in a potent antigen-specific CTL response and virus protection.

Figure 52 shows immunostimulatory nucleic acids packaged in VLPs are even more efficient in inducing CTL responses than VLPs mixed with immunostimulatory nucleic acids.

Figure 53 shows analysis of non-enzymatic RNA hydrolysis of the RNA in Q $\beta$  VLPs.

Figure 54 shows packaging of oligodeoxynucleotides into Q $\beta$  VLPs after non-enzymatic RNA hydrolysis.

Figure 55 shows analysis of packaging of oligodeoxynucleotides into Q $\beta$  VLPs after non-enzymatic RNA hydrolysis.

### ***Detailed Description Of The Invention***

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are hereinafter described.

#### **1. Definitions**

Amino acid linker: An "amino acid linker", or also just termed "linker" within this specification, as used herein, either associates the antigen or antigenic determinant with the second attachment site, or more preferably, already comprises or contains the second attachment site, typically - but not

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necessarily - as one amino acid residue, preferably as a cysteine residue. The term "amino acid linker" as used herein, however, does not intend to imply that such an amino acid linker consists exclusively of amino acid residues, even if an amino acid linker consisting of amino acid residues is a preferred embodiment of the present invention. The amino acid residues of the amino acid linker are, preferably, composed of naturally occurring amino acids or unnatural amino acids known in the art, all-L or all-D or mixtures thereof. However, an amino acid linker comprising a molecule with a sulfhydryl group or cysteine residue is also encompassed within the invention. Such a molecule comprise preferably a C1-C6 alkyl-, cycloalkyl (C5,C6), aryl or heteroaryl moiety. However, in addition to an amino acid linker, a linker comprising preferably a C1-C6 alkyl-, cycloalkyl- (C5,C6), aryl- or heteroaryl- moiety and devoid of any amino acid(s) shall also be encompassed within the scope of the invention. Association between the antigen or antigenic determinant or optionally the second attachment site and the amino acid linker is preferably by way of at least one covalent bond, more preferably by way of at least one peptide bond.

**Animal:** As used herein, the term "animal" is meant to include, for example, humans, sheep, horses, cattle, pigs, dogs, cats, rats, mice, mammals, birds, reptiles, fish, insects and arachnids.

**Antibody:** As used herein, the term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant. The term is meant to include whole antibodies and antigen-binding fragments thereof, including single-chain antibodies. Most preferably the antibodies are human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V<sub>L</sub> or V<sub>H</sub> domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit, goat, guinea pig, camel, horse or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and

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include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

Antigen: As used herein, the term "antigen" refers to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T- epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens.

A "microbial antigen" as used herein is an antigen of a microorganism and includes, but is not limited to, infectious virus, infectious bacteria, parasites and infectious fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic or recombinant compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to the skilled artisan.

Examples of infectious viruses that have been found in humans include but are not limited to: Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III); and other isolates, such as HIV-LP); Picornaviridae (e.g. polio

viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bunyaviridae (e.g. Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, *Pasteurella* species, *Staphylococci* species and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pylori*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps. (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus viridans*

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group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, *Actinomyces israeli* and *Chlamydia*.

Examples of infectious fungi include: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis* and *Candida albicans*. Other infectious organisms (i.e., protists) include: *Plasmodium* such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, *Toxoplasma gondii* and *Shistosoma*.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C. G. A. Thomas, "Medical Microbiology", Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

The compositions and methods of the invention are also useful for treating cancer by stimulating an antigen-specific immune response against a cancer antigen. A "tumor antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer and which is capable of provoking an immune response. In particular, the compound is capable of provoking an immune response when presented in the context of an MHC molecule. Tumor antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, *et al.*, *Cancer Research*, 54:1055 (1994), by partially purifying the antigens, by recombinant technology or by de novo synthesis of known antigens. Tumor antigens include antigens that are antigenic portions of or are a whole tumor or cancer polypeptide. Such antigens can be isolated or prepared recombinantly or by



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any other means known in the art. Cancers or tumors include, but are not limited to, biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas.

**Antigenic determinant:** As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediator of cellular immunity. Thus, antigenic determinants or epitopes are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptors.

**Antigen presenting cell:** As used herein, the term "antigen presenting cell" is meant to refer to a heterogenous population of leucocytes or bone marrow derived cells which possess an immunostimulatory capacity. For example, these cells are capable of generating peptides bound to MHC molecules that can be recognized by T cells. The term is synonymous with the term "accessory cell" and includes, for example, Langerhans' cells, interdigitating cells, B cells, macrophages and dendritic cells. Under some conditions, epithelial cells, endothelial cells and other, non-bone marrow derived cells may also serve as antigen presenting cells.

**Association:** As used herein, the term "association" as it applies to the first and second attachment sites, refers to the binding of the first and second attachment sites that is preferably by way of at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof, preferably the nature of the association is covalent.

**Attachment Site, First:** As used herein, the phrase "first attachment site" refers to an element of non-natural or natural origin, to which the second

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attachment site located on the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, an amino acid, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. The first attachment site is located, typically and preferably on the surface, of the virus-like particle. Multiple first attachment sites are present on the surface of virus-like particle typically in a repetitive configuration.

Attachment Site, Second: As used herein, the phrase "second attachment site" refers to an element associated with the antigen or antigenic determinant to which the first attachment site located on the surface of the virus-like particle may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant. The term "antigen or antigenic determinant with at least one second attachment site" refers, therefore, to an antigen or antigenic construct comprising at least the antigen or antigenic determinant and the second attachment site. However, in particular for a second attachment site, which is of non-natural origin, i.e. not naturally occurring within the antigen or antigenic determinant, these antigen or antigenic constructs comprise an "amino acid linker".

Bound: As used herein, the term "bound" refers to binding that may be covalent, *e.g.*, by chemically coupling, or non-covalent, *e.g.*, ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term also includes the enclosement, or partial enclosement, of a substance. The term "bound" is

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broadier than and includes terms such as "coupled," "fused," "enclosed", "packaged" and "attached." For example, the immunostimulatory substance such as the unmethylated CpG-containing oligonucleotide can be enclosed by the VLP without the existence of an actual binding, neither covalently nor non-covalently.

**Coat protein(s):** As used herein, the term "coat protein(s)" refers to the protein(s) of a bacteriophage or a RNA-phage capable of being incorporated within the capsid assembly of the bacteriophage or the RNA-phage. However, when referring to the specific gene product of the coat protein gene of RNA-phages the term "CP" is used. For example, the specific gene product of the coat protein gene of RNA-phage Q $\beta$  is referred to as "Q $\beta$  CP", whereas the "coat proteins" of bacteriophage Q $\beta$  comprise the "Q $\beta$  CP" as well as the A1 protein. The capsid of Bacteriophage Q $\beta$  is composed mainly of the Q $\beta$  CP, with a minor content of the A1 protein. Likewise, the VLP Q $\beta$  coat protein contains mainly Q $\beta$  CP, with a minor content of A1 protein.

**Coupled:** As used herein, the term "coupled" refers to attachment by covalent bonds or by strong non-covalent interactions. Any method normally used by those skilled in the art for the coupling of biologically active materials can be used in the present invention.

**Fusion:** As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, *i.e.*, insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.

**CpG:** As used herein, the term "CpG" refers to an oligonucleotide which contains an unmethylated cytosine, guanine dinucleotide sequence (*e.g.* "CpG DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates/activates, *e.g.* has a mitogenic effect on, or induces or increases cytokine expression by, a vertebrate cell. For example, CpGs can be useful in activating B cells, NK cells and antigen-presenting cells, such as monocytes, dendritic cells and macrophages, and T cells. The

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CpGs can include nucleotide analogs such as analogs containing phosphorothioester bonds and can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immune activity.

**Epitope:** As used herein, the term "epitope" refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. An "immunogenic epitope," as used herein, is defined as a portion of a polypeptide that elicits an antibody response or induces a T-cell response in an animal, as determined by any method known in the art. (See, for example, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983)). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic. Antigenic epitopes can also be T-cell epitopes, in which case they can be bound immunospecifically by a T-cell receptor within the context of an MHC molecule.

An epitope can comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally, an epitope consists of at least about 5 such amino acids, and more usually, consists of at least about 8-10 such amino acids. If the epitope is an organic molecule, it may be as small as Nitrophenyl.

**Immune response:** As used herein, the term "immune response" refers to a humoral immune response and/or cellular immune response leading to the activation or proliferation of B- and/or T-lymphocytes. In some instances, however, the immune responses may be of low intensity and become detectable only when using at least one substance in accordance with the invention. "Immunogenic" refers to an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. An

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"immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant.

Immunization: As used herein, the terms "immunize" or "immunization" or related terms refer to conferring the ability to mount a substantial immune response (comprising antibodies or cellular immunity such as effector CTL) against a target antigen or epitope. These terms do not require that complete immunity be created, but rather that an immune response be produced which is substantially greater than baseline. For example, a mammal may be considered to be immunized against a target antigen if the cellular and/or humoral immune response to the target antigen occurs following the application of methods of the invention.

Immunostimulatory nucleic acid: As used herein, the term immunostimulatory nucleic acid refers to a nucleic acid capable of inducing and/or enhancing an immune response. Immunostimulatory nucleic acids, as used herein, comprise ribonucleic acids and in particular deoxyribonucleic acids. Preferably, immunostimulatory nucleic acids contain at least one CpG motif e.g. a CG dinucleotide in which the C is unmethylated. The CG dinucleotide can be part of a palindromic sequence or can be encompassed within a non-palindromic sequence. Immunostimulatory nucleic acids not containing CpG motifs as described above encompass, by way of example, nucleic acids lacking CpG dinucleotides, as well as nucleic acids containing CG motifs with a methylated CG dinucleotide. The term "immunostimulatory nucleic acid" as used herein should also refer to nucleic acids that contain modified bases such as 4-bromo-cytosine.

Immunostimulatory substance: As used herein, the term "immunostimulatory substance" refers to a substance capable of inducing and/or enhancing an immune response. Immunostimulatory substances, as used herein, include, but are not limited to, toll-like receptor activating substances and substances inducing cytokine secretion. Toll-like receptor activating substances include, but are not limited to, immunostimulatory

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nucleic acids, peptideoglycans, lipopolysaccharides, lipoteichonic acids, imidazoquinoline compounds, flagellins, lipoproteins, and immunostimulatory organic substances such as taxol.

Natural origin: As used herein, the term "natural origin" means that the whole or parts thereof are not synthetic and exist or are produced in nature.

Non-natural: As used herein, the term generally means not from nature, more specifically, the term means from the hand of man.

Non-natural origin: As used herein, the term "non-natural origin" generally means synthetic or not from nature; more specifically, the term means from the hand of man.

Ordered and repetitive antigen or antigenic determinant array: As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a typically and preferably uniform spacial arrangement of the antigens or antigenic determinants with respect to the core particle and virus-like particle, respectively. In one embodiment of the invention, the repeating pattern may be a geometric pattern. Typical and preferred examples of suitable ordered and repetitive antigen or antigenic determinant arrays are those which possess strictly repetitive paracrystalline orders of antigens or antigenic determinants, preferably with spacings of 0.5 to 30 nanometers, more preferably 5 to 15 nanometers.

Oligonucleotide: As used herein, the terms "oligonucleotide" or "oligomer" refer to a nucleic acid sequence comprising 2 or more nucleotides, generally at least about 6 nucleotides to about 100,000 nucleotides, preferably about 6 to about 2000 nucleotides, and more preferably about 6 to about 300 nucleotides, even more preferably about 20 to about 300 nucleotides, and even more preferably about 20 to about 100 nucleotides. The terms "oligonucleotide" or "oligomer" also refer to a nucleic acid sequence comprising more than 100 to about 2000 nucleotides, preferably more than 100 to about 1000 nucleotides, and more preferably more than 100 to about 500 nucleotides. "Oligonucleotide" also generally refers to any

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polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Oligonucleotide" includes, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "oligonucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. Further, an oligonucleotide can be synthetic, genomic or recombinant, *e.g.*,  $\lambda$ -DNA, cosmid DNA, artificial bacterial chromosome, yeast artificial chromosome and filamentous phage such as M13.

The term "oligonucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. For example, suitable nucleotide modifications/analogs include peptide nucleic acid, inosin, tritylated bases, phosphorothioates, alkylphosphorothioates, 5-nitroindole deoxyribofuranosyl, 5-methyldeoxycytosine and 5,6-dihydro-5,6-dihydroxydeoxythymidine. A variety of modifications have been made to DNA and RNA; thus, "oligonucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. Other nucleotide analogs/modifications will be evident to those skilled in the art.

**Packaged:** The term "packaged" as used herein refers to the state of an immunostimulatory substance, in particular an immunostimulatory nucleic acid in relation to the VLP. The term "packaged" as used herein includes binding that may be covalent, *e.g.*, by chemically coupling, or non-covalent, *e.g.*, ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term also includes the enclosement, or partial enclosement, of a substance.

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The term "packaged" includes terms such as "coupled," "enclosed" and "attached." For example, the immunostimulatory substance such as the unmethylated CpG-containing oligonucleotide can be enclosed by the VLP without the existence of an actual binding, neither covalently nor non-covalently. In preferred embodiments, in particular, if immunostimulatory nucleic acids are the immunostimulatory substances, the term "packaged" indicates that the nucleic acid in a packaged state is not accessible to DNase or RNase hydrolysis. In preferred embodiments, the immunostimulatory nucleic acid is packaged inside the VLP capsids, most preferably in a non-covalent manner.

The compositions of the invention can be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application.

**Organic molecule:** As used herein, the term "organic molecule" refers to any chemical entity of natural or synthetic origin. In particular the term "organic molecule" as used herein encompasses, for example, any molecule being a member of the group of nucleotides, lipids, carbohydrates, polysaccharides, lipopolysaccharides, steroids, alkaloids, terpenes and fatty acids, being either of natural or synthetic origin. In particular, the term "organic molecule" encompasses molecules such as nicotine, cocaine, heroin or other pharmacologically active molecules contained in drugs of abuse. In general an organic molecule contains or is modified to contain a chemical functionality allowing its coupling, binding or other method of attachment to the virus-like particle in accordance with the invention.

**Polypeptide:** As used herein, the term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). It indicates a molecular chain of amino



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acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to post-expression modifications of the polypeptide, for example, glycosolations, acetylations, phosphorylations, and the like. A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence. It may also be generated in any manner, including chemical synthesis.

A substance which "enhances" an immune response refers to a substance in which an immune response is observed that is greater or intensified or deviated in any way with the addition of the substance when compared to the same immune response measured without the addition of the substance. For example, the lytic activity of cytotoxic T cells can be measured, *e.g.* using a  $^{51}\text{Cr}$  release assay, with and without the substance. The amount of the substance at which the CTL lytic activity is enhanced as compared to the CTL lytic activity without the substance is said to be an amount sufficient to enhance the immune response of the animal to the antigen. In a preferred embodiment, the immune response is enhanced by a factor of at least about 2, more preferably by a factor of about 3 or more. The amount of cytokines secreted may also be altered.

**Effective Amount:** As used herein, the term "effective amount" refers to an amount necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves this selected result, and such an amount could be determined as a matter of routine by a person skilled in the art. For example, an effective amount for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to antigen. The term is also synonymous with "sufficient amount."

The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the size of the subject, and/or the severity of

the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular composition of the present invention without necessitating undue experimentation.

Self antigen: As used herein, the term "self antigen" refers to proteins encoded by the host's DNA and products generated by proteins or RNA encoded by the host's DNA are defined as self. In addition, proteins that result from a combination of two or several self-molecules or that represent a fraction of a self-molecule and proteins that have a high homology to self-molecules as defined above (>95%, preferably >97%, more preferably >99%) may also be considered self. In a further preferred embodiment of the present invention, the antigen is a self antigen. Very preferred embodiments of self-antigens useful for the present invention are described WO 02/056905, the disclosures of which are herewith incorporated by reference in its entirety.

Treatment: As used herein, the terms "treatment", "treat", "treated" or "treating" refer to prophylaxis and/or therapy. When used with respect to an infectious disease, for example, the term refers to a prophylactic treatment which increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen or will show signs of illness attributable to the infection, as well as a treatment after the subject has become infected in order to fight the infection, *e.g.*, reduce or eliminate the infection or prevent it from becoming worse.

Vaccine: As used herein, the term "vaccine" refers to a formulation which contains the composition of the present invention and which is in a form that is capable of being administered to an animal. Typically, the vaccine comprises a conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat a condition. Upon introduction into a host, the vaccine is able to provoke an immune response including, but not

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limited to, the production of antibodies, cytokines and/or other cellular responses.

Optionally, the vaccine of the present invention additionally includes an adjuvant which can be present in either a minor or major proportion relative to the compound of the present invention. The term "adjuvant" as used herein refers to non-specific stimulators of the immune response or substances that allow generation of a depot in the host which when combined with the vaccine of the present invention provide for an even more enhanced immune response. A variety of adjuvants can be used. Examples include incomplete Freund's adjuvant, aluminum hydroxide and modified muramyl dipeptide. The term "adjuvant" as used herein also refers to typically specific stimulators of the immune response which when combined with the vaccine of the present invention provide for an even more enhanced and typically specific immune response. Examples include, but limited to, GM-CSF, IL-2, IL-12, IFN $\alpha$ . Further examples are within the knowledge of the person skilled in the art.

Virus-like particle: As used herein, the term "virus-like particle" refers to a structure resembling a virus particle but which has not been demonstrated to be pathogenic. Typically, a virus-like particle in accordance with the invention does not carry genetic information encoding for the proteins of the virus-like particle. In general, virus-like particles lack the viral genome and, therefore, are noninfectious. Also, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified. Some virus-like particles may contain nucleic acid distinct from their genome. As indicated, a virus-like particle in accordance with the invention is non-replicative and noninfectious since it lacks all or part of the viral genome, in particular the replicative and infectious components of the viral genome. A virus-like particle in accordance with the invention may contain nucleic acid distinct from their genome. A typical and preferred embodiment of a virus-like particle in accordance with the present invention is a viral capsid such as the viral capsid of the corresponding virus, bacteriophage, or RNA-phage. The terms "viral capsid" or "capsid", as interchangeably used herein, refer to a

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macromolecular assembly composed of viral protein subunits. Typically and preferably, the viral protein subunits assemble into a viral capsid and capsid, respectively, having a structure with an inherent repetitive organization, wherein said structure is, typically, spherical or tubular. For example, the capsids of RNA-phages or HBcAg's have a spherical form of icosahedral symmetry. The term "capsid-like structure" as used herein, refers to a macromolecular assembly composed of viral protein subunits resembling the capsid morphology in the above defined sense but deviating from the typical symmetrical assembly while maintaining a sufficient degree of order and repetitiveness.

Virus-like particle of a bacteriophage: As used herein, the term "virus-like particle of a bacteriophage" refers to a virus-like particle resembling the structure of a bacteriophage, being non replicative and noninfectious, and lacking at least the gene or genes encoding for the replication machinery of the bacteriophage, and typically also lacking the gene or genes encoding the protein or proteins responsible for viral attachment to or entry into the host. This definition should, however, also encompass virus-like particles of bacteriophages, in which the aforementioned gene or genes are still present but inactive, and, therefore, also leading to non-replicative and noninfectious virus-like particles of a bacteriophage.

VLP of RNA phage coat protein: The capsid structure formed from the self-assembly of 180 subunits of RNA phage coat protein and optionally containing host RNA is referred to as a "VLP of RNA phage coat protein". A specific example is the VLP of Q $\beta$  coat protein. In this particular case, the VLP of Q $\beta$  coat protein may either be assembled exclusively from Q $\beta$  CP subunits (generated by expression of a Q $\beta$  CP gene containing, for example, a TAA stop codon precluding any expression of the longer A1 protein through suppression, see Kozlovskaya, T.M., *et al.*, *Intervirology* 39: 9-15 (1996)), or additionally contain A1 protein subunits in the capsid assembly.

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The term "virus particle" as used herein refers to the morphological form of a virus. In some virus types it comprises a genome surrounded by a protein capsid; others have additional structures (*e.g.*, envelopes, tails, etc.).

Non-enveloped viral particles are made up of a proteinaceous capsid that surrounds and protects the viral genome. Enveloped viruses also have a capsid structure surrounding the genetic material of the virus but, in addition, have a lipid bilayer envelope that surrounds the capsid. In a preferred embodiment of the invention, the VLP's are free of a lipoprotein envelope or a lipoprotein-containing envelope. In a further preferred embodiment, the VLP's are free of an envelope altogether.

One, a, or an: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning, polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies are well known to those skilled in the art and can be conveniently found in published laboratory methods manuals (*e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)). Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth. Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., "Protein Purification Principles and Practice," 3<sup>rd</sup> ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

## 2. Compositions and Methods for Enhancing an Immune Response

The disclosed invention provides compositions and methods for enhancing an immune response against one or more antigens in an animal. Compositions of the invention comprise, or alternatively consist of, a virus-like particle and an immunostimulatory substance, preferably an immunostimulatory nucleic acid, and even more preferably an unmethylated CpG-containing oligonucleotide where the an immunostimulatory substance, the immunostimulatory nucleic acid or the oligonucleotide is bound to the virus-like particle. Furthermore, the invention conveniently enables the practitioner to construct such a composition for various treatment and/or prophylactic prevention purposes, which include the prevention and/or treatment of infectious diseases, as well as chronic infectious diseases, and the prevention and/or treatment of cancers, for example.

Virus-like particles in the context of the present application refer to structures resembling a virus particle but which are not pathogenic. In general, virus-like particles lack the viral genome and, therefore, are noninfectious. Also, virus-like particles can be produced in large quantities by heterologous expression and can be easily purified.

In a preferred embodiment, the virus-like particle is a recombinant virus-like particle. The skilled artisan can produce VLPs using recombinant DNA technology and virus coding sequences which are readily available to the public. For example, the coding sequence of a virus envelope or core protein can be engineered for expression in a baculovirus expression vector using a commercially available baculovirus vector, under the regulatory control of a virus promoter, with appropriate modifications of the sequence to allow functional linkage of the coding sequence to the regulatory sequence. The coding sequence of a virus envelope or core protein can also be engineered for expression in a bacterial expression vector, for example.

Examples of VLPs include, but are not limited to, the capsid proteins of Hepatitis B virus (Ulrich, *et al.*, *Virus Res.* 50:141-182 (1998)), measles

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virus (Warnes, *et al.*, *Gene* 160:173-178 (1995)), Sindbis virus, rotavirus (U.S. Patent Nos. 5,071,651 and 5,374,426), foot-and-mouth-disease virus (Twomey, *et al.*, *Vaccine* 13:1603-1610, (1995)), Norwalk virus (Jiang, X., *et al.*, *Science* 250:1580-1583 (1990); Matsui, S.M., *et al.*, *J. Clin. Invest.* 87:1456-1461 (1991)), the retroviral GAG protein (PCT Patent Appl. No. WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291), human papilloma virus (WO 98/15631), human polyoma virus (Sasnauskas K., *et al.*, *Biol. Chem.* 380(3):381-386 (1999); Sasnauskas K., *et al.*, *Generation of recombinant virus-like particles of different polyomaviruses in yeast*. 3<sup>rd</sup> International Workshop "Virus-like particles as vaccines." Berlin, September 26-29 (2001)), RNA phages, Ty, fr-phage, GA-phage, AP 205-phage and, in particular, Q $\beta$ -phage.

As will be readily apparent to those skilled in the art, the VLP of the invention is not limited to any specific form. The particle can be synthesized chemically or through a biological process, which can be natural or non-natural. By way of example, this type of embodiment includes a virus-like particle or a recombinant form thereof. In a more specific embodiment, the VLP can comprise, or alternatively consist of, recombinant polypeptides of Rotavirus; recombinant polypeptides of Norwalk virus; recombinant polypeptides of Alphavirus; recombinant proteins which form bacterial pili or pilus-like structures; recombinant polypeptides of Foot and Mouth Disease virus; recombinant polypeptides of measles virus, recombinant polypeptides of Sindbis virus, recombinant polypeptides of Retrovirus; recombinant polypeptides of Hepatitis B virus (*e.g.*, a HBcAg); recombinant polypeptides of Tobacco mosaic virus; recombinant polypeptides of Flock House Virus; recombinant polypeptides of human Papillomavirus; recombinant polypeptides of Polyoma virus and, in particular, recombinant polypeptides of human Polyoma virus, and in particular recombinant polypeptides of BK virus; recombinant polypeptides of bacteriophages, recombinant polypeptides of RNA phages; recombinant polypeptides of Ty; recombinant polypeptides of fr-phage, recombinant polypeptides of GA-phage, recombinant polypeptides

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of AP 205-phage and, in particular, recombinant polypeptides of Q $\beta$ -phage. The virus-like particle can further comprise, or alternatively consist of, one or more fragments of such polypeptides, as well as variants of such polypeptides. Variants of polypeptides can share, for example, at least 80%, 85%, 90%, 95%, 97%, or 99% identity at the amino acid level with their wild-type counterparts.

In a preferred embodiment, the virus-like particle comprises, consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage. Preferably, the RNA-phage is selected from the group consisting of a) bacteriophage Q $\beta$ ; b) bacteriophage R17; c) bacteriophage fr; d) bacteriophage GA; e) bacteriophage SP; f) bacteriophage MS2; g) bacteriophage M11; h) bacteriophage MX1; i) bacteriophage NL95; k) bacteriophage f2; and l) bacteriophage PP7.

In another preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of the RNA-bacteriophage Q $\beta$  or of the RNA-bacteriophage fr.

In a further preferred embodiment of the present invention, the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins of RNA phages.

RNA-phage coat proteins forming capsids or VLPs, or fragments of the bacteriophage coat proteins compatible with self-assembly into a capsid or a VLP, are, therefore, further preferred embodiments of the present invention. Bacteriophage Q $\beta$  coat proteins, for example, can be expressed recombinantly in *E. coli*. Further, upon such expression these proteins spontaneously form capsids. Additionally, these capsids form a structure with an inherent repetitive organization.

Specific preferred examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA bacteriophages such as bacteriophage Q $\beta$  (SEQ ID NO:10; PIR Database, Accession No. VCBPQ $\beta$  referring to Q $\beta$  CP and SEQ ID NO: 11;



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Accession No. AAA16663 referring to Q $\beta$  A1 protein), bacteriophage R17 (SEQ ID NO:12; PIR Accession No. VCBPR7), bacteriophage fr (SEQ ID NO:13; PIR Accession No. VCBPFR), bacteriophage GA (SEQ ID NO:14; GenBank Accession No. NP-040754), bacteriophage SP (SEQ ID NO:15; GenBank Accession No. CAA30374 referring to SP CP and SEQ ID NO: 16; Accession No. referring to SP A1 protein), bacteriophage MS2 (SEQ ID NO:17; PIR Accession No. VCBPM2), bacteriophage M11 (SEQ ID NO:18; GenBank Accession No. AAC06250), bacteriophage MX1 (SEQ ID NO:19; GenBank Accession No. AAC14699), bacteriophage NL95 (SEQ ID NO:20; GenBank Accession No. AAC14704), bacteriophage f2 (SEQ ID NO: 21; GenBank Accession No. P03611), bacteriophage PP7 (SEQ ID NO: 22). Furthermore, the A1 protein of bacteriophage Q $\beta$  or C-terminal truncated forms missing as much as 100, 150 or 180 amino acids from its C-terminus may be incorporated in a capsid assembly of Q $\beta$  coat proteins. Generally, the percentage of Q $\beta$  A1 protein relative to Q $\beta$  CP in the capsid assembly will be limited, in order to ensure capsid formation.

Q $\beta$  coat protein has also been found to self-assemble into capsids when expressed in *E. coli* (Kozlovska TM. *et al.*, *GENE* 137: 133-137 (1993)). The obtained capsids or virus-like particles showed an icosahedral phage-like capsid structure with a diameter of 25 nm and T=3 quasi symmetry. Further, the crystal structure of phage Q $\beta$  has been solved. The capsid contains 180 copies of the coat protein, which are linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, R. *et al.*, *Structure* 4: 543-5554 (1996)) leading to a remarkable stability of the capsid of Q $\beta$  coat protein. Capsids or VLPs made from recombinant Q $\beta$  coat protein may contain, however, subunits not linked via disulfide links to other subunits within the capsid, or incompletely linked. Thus, upon loading recombinant Q $\beta$  capsid on non-reducing SDS-PAGE, bands corresponding to monomeric Q $\beta$  coat protein as well as bands corresponding to the hexamer or pentamer of Q $\beta$  coat protein are visible. Incompletely disulfide-linked subunits could appear as dimer,

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trimer or even tetramer bands in non-reducing SDS-PAGE. Q $\beta$  capsid protein also shows unusual resistance to organic solvents and denaturing agents. Surprisingly, we have observed that DMSO and acetonitrile concentrations as high as 30%, and Guanidinium concentrations as high as 1 M do not affect the stability of the capsid. The high stability of the capsid of Q $\beta$  coat protein is an advantageous feature, in particular, for its use in immunization and vaccination of mammals and humans in accordance of the present invention.

Upon expression in *E. coli*, the N-terminal methionine of Q $\beta$  coat protein is usually removed, as we observed by N-terminal Edman sequencing as described in Stoll, E. et al. *J. Biol. Chem.* 252:990-993 (1977). VLP composed from Q $\beta$  coat proteins where the N-terminal methionine has not been removed, or VLPs comprising a mixture of Q $\beta$  coat proteins where the N-terminal methionine is either cleaved or present are also within the scope of the present invention.

Further RNA phage coat proteins have also been shown to self-assemble upon expression in a bacterial host (Kastelein, RA. *et al.*, *Gene* 23: 245-254 (1983), Kozlovskaya, TM. *et al.*, *Dokl. Akad. Nauk SSSR* 287: 452-455 (1986), Adhin, MR. *et al.*, *Virology* 170: 238-242 (1989), Ni, CZ., *et al.*, *Protein Sci.* 5: 2485-2493 (1996), Priano, C. et al., *J. Mol. Biol.* 249: 283-297 (1995)). The Q $\beta$  phage capsid contains, in addition to the coat protein, the so called read-through protein A1 and the maturation protein A2. A1 is generated by suppression at the UGA stop codon and has a length of 329 aa. The capsid of phage Q $\beta$  recombinant coat protein used in the invention is devoid of the A2 lysis protein, and contains RNA from the host. The coat protein of RNA phages is an RNA binding protein, and interacts with the stem loop of the ribosomal binding site of the replicase gene acting as a translational repressor during the life cycle of the virus. The sequence and structural elements of the interaction are known (Witherell, GW. & Uhlenbeck, OC. *Biochemistry* 28: 71-76 (1989); Lim F. et al., *J. Biol. Chem.* 271: 31839-31845 (1996)). The

stem loop and RNA in general are known to be involved in the virus assembly (Golmohammadi, R. *et al.*, *Structure* 4: 543-5554 (1996)).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage, wherein the recombinant proteins comprise, consist essentially of or alternatively consist of mutant coat proteins of a RNA phage, preferably of mutant coat proteins of the RNA phages mentioned above. In another preferred embodiment, the mutant coat proteins of the RNA phage have been modified by removal of at least one lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution; alternatively, the mutant coat proteins of the RNA phage have been modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

In another preferred embodiment, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of the RNA-bacteriophage Q $\beta$ , wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins having an amino acid sequence of SEQ ID NO:10, or a mixture of coat proteins having amino acid sequences of SEQ ID NO:10 and of SEQ ID NO: 11 or mutants of SEQ ID NO: 11 and wherein the N-terminal methionine is preferably cleaved.

In a further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of Q $\beta$ , or fragments thereof, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of mutant Q $\beta$  coat proteins. In another preferred embodiment, these mutant coat proteins have been modified by removal of at least one lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution. Alternatively, these mutant coat proteins have been

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modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

Four lysine residues are exposed on the surface of the capsid of Q $\beta$  coat protein. Q $\beta$  mutants, for which exposed lysine residues are replaced by arginines can also be used for the present invention. The following Q $\beta$  coat protein mutants and mutant Q $\beta$  VLPs can, thus, be used in the practice of the invention: "Q $\beta$ -240" (Lys13-Arg; SEQ ID NO:23), "Q $\beta$ -243" (Asn 10-Lys; SEQ ID NO:24), "Q $\beta$ -250" (Lys 2-Arg, Lys13-Arg; SEQ ID NO:25), "Q $\beta$ -251" (SEQ ID NO:26) and "Q $\beta$ -259" (Lys 2-Arg, Lys16-Arg; SEQ ID NO:27). Thus, in further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of mutant Q $\beta$  coat proteins, which comprise proteins having an amino acid sequence selected from the group of a) the amino acid sequence of SEQ ID NO: 23; b) the amino acid sequence of SEQ ID NO:24; c) the amino acid sequence of SEQ ID NO: 25; d) the amino acid sequence of SEQ ID NO:26; and e) the amino acid sequence of SEQ ID NO: 27. The construction, expression and purification of the above indicated Q $\beta$  coat proteins, mutant Q $\beta$  coat protein VLPs and capsids, respectively, are disclosed in pending U.S. Application No. 10/050,902 filed on January 18, 2002. In particular is hereby referred to Example 18 of above mentioned application.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins of Q $\beta$ , or fragments thereof, wherein the recombinant proteins comprise, consist essentially of or alternatively consist of a mixture of either one of the foregoing Q $\beta$  mutants and the corresponding A1 protein.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant proteins, or fragments thereof, of RNA-phage AP205.

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The AP205 genome consists of a maturation protein, a coat protein, a replicase and two open reading frames not present in related phages; a lysis gene and an open reading frame playing a role in the translation of the maturation gene (Klovins, J., *et al.*, *J. Gen. Virol.* 83: 1523-33 (2002)). AP205 coat protein can be expressed from plasmid pAP283-58 (SEQ ID NO: 79), which is a derivative of pQb10 (Kozlovskaya, T. M. *et al.*, *Gene* 137:133-37 (1993)), and which contains an AP205 ribosomal binding site. Alternatively, AP205 coat protein may be cloned into pQb185, downstream of the ribosomal binding site present in the vector. Both approaches lead to expression of the protein and formation of capsids as described in the co-pending US provisional patent application with the title "Molecular Antigen Arrays" (Application No. 60/396,126) and having been filed on July 17, 2002, which is incorporated by reference in its entirety. Vectors pQb10 and pQb185 are vectors derived from pGEM vector, and expression of the cloned genes in these vectors is controlled by the *trp* promoter (Kozlovskaya, T. M. *et al.*, *Gene* 137:133-37 (1993)). Plasmid pAP283-58 (SEQ ID NO:79) comprises a putative AP205 ribosomal binding site in the following sequence, which is downstream of the XbaI site, and immediately upstream of the ATG start codon of the AP205 coat protein: *tctaga*ATTTTCTGCGCACCCAT CCCGGGTGGCGCCCAAAGTGAGGAAAATCAGatg. The vector pQb185 comprises a Shine Delagarno sequence downstream from the XbaI site and upstream of the start codon (*tctaga*TTAACCCAACGCGTAGGAGTCAGGCCatg, Shine Delagarno sequence underlined).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant coat proteins, or fragments thereof, of the RNA-phage AP205.

This preferred embodiment of the present invention, thus, comprises AP205 coat proteins that form capsids. Such proteins are recombinantly expressed, or prepared from natural sources. AP205 coat proteins produced in bacteria spontaneously form capsids, as evidenced by Electron Microscopy

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(EM) and immunodiffusion. The structural properties of the capsid formed by the AP205 coat protein (SEQ ID NO: 80) and those formed by the coat protein of the AP205 RNA phage are nearly indistinguishable when seen in EM. AP205 VLPs are highly immunogenic, and can be linked with antigens and/or antigenic determinants to generate vaccine constructs displaying the antigens and/or antigenic determinants oriented in a repetitive manner. High titers are elicited against the so displayed antigens showing that bound antigens and/or antigenic determinants are accessible for interacting with antibody molecules and are immunogenic.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

Assembly-competent mutant forms of AP205 VLPs, including AP205 coat protein with the substitution of proline at amino acid 5 to threonine (SEQ ID NO: 81), may also be used in the practice of the invention and leads to a further preferred embodiment of the invention. These VLPs, AP205 VLPs derived from natural sources, or AP205 viral particles, may be bound to antigens to produce ordered repetitive arrays of the antigens in accordance with the present invention.

AP205 P5-T mutant coat protein can be expressed from plasmid pAP281-32 (SEQ ID No. 82), which is derived directly from pQb185, and which contains the mutant AP205 coat protein gene instead of the Q $\beta$  coat protein gene. Vectors for expression of the AP205 coat protein are transfected into *E. coli* for expression of the AP205 coat protein.

Methods for expression of the coat protein and the mutant coat protein, respectively, leading to self-assembly into VLPs are described in co-pending US provisional patent application with the title "Molecular Antigen Arrays" (Application No. 60/396,126) and having been filed on July 17, 2002, which is incorporated by reference in its entirety. Suitable *E. coli* strains include, but are not limited to, *E. coli* K802, JM 109, RR1. Suitable vectors and strains and

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combinations thereof can be identified by testing expression of the coat protein and mutant coat protein, respectively, by SDS-PAGE and capsid formation and assembly by optionally first purifying the capsids by gel filtration and subsequently testing them in an immunodiffusion assay (Ouchterlony test) or Electron Microscopy (Kozłowska, T. M. *et al.*, *Gene* 137:133-37 (1993)).

AP205 coat proteins expressed from the vectors pAP283-58 and pAP281-32 may be devoid of the initial Methionine amino-acid, due to processing in the cytoplasm of *E. coli*. Cleaved, uncleaved forms of AP205 VLP, or mixtures thereof are further preferred embodiments of the invention.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of a mixture of recombinant coat proteins, or fragments thereof, of the RNA-phage AP205 and of recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of fragments of recombinant coat proteins or recombinant mutant coat proteins of the RNA-phage AP205.

Recombinant AP205 coat protein fragments capable of assembling into a VLP and a capsid, respectively are also useful in the practice of the invention. These fragments may be generated by deletion, either internally or at the termini of the coat protein and mutant coat protein, respectively. Insertions in the coat protein and mutant coat protein sequence or fusions of antigen sequences to the coat protein and mutant coat protein sequence, and compatible with assembly into a VLP, are further embodiments of the invention and lead to chimeric AP205 coat proteins, and particles, respectively. The outcome of insertions, deletions and fusions to the coat protein sequence and whether it is compatible with assembly into a VLP can be determined by electron microscopy.

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The particles formed by the AP205 coat protein, coat protein fragments and chimeric coat proteins described above, can be isolated in pure form by a combination of fractionation steps by precipitation and of purification steps by gel filtration using *e.g.* Sepharose CL-4B, Sepharose CL-2B, Sepharose CL-6B columns and combinations thereof as described in the co-pending US provisional patent application with the title "Molecular Antigen Arrays" (Application No. 60/396,126) and having been filed on July 17, 2002, which is incorporated by reference in its entirety. Other methods of isolating virus-like particles are known in the art, and may be used to isolate the virus-like particles (VLPs) of bacteriophage AP205. For example, the use of ultracentrifugation to isolate VLPs of the yeast retrotransposon Ty is described in U.S. Patent No. 4,918,166, which is incorporated by reference herein in its entirety.

The crystal structure of several RNA bacteriophages has been determined (Golmohammadi, R. *et al.*, *Structure* 4:543-554 (1996)). Using such information, surface exposed residues can be identified and, thus, RNA-phage coat proteins can be modified such that one or more reactive amino acid residues can be inserted by way of insertion or substitution. As a consequence, those modified forms of bacteriophage coat proteins can also be used for the present invention. Thus, variants of proteins which form capsids or capsid-like structures (*e.g.*, coat proteins of bacteriophage Q $\beta$ , bacteriophage R17, bacteriophage fr, bacteriophage GA, bacteriophage SP, and bacteriophage MS2, bacteriophage AP 205) can also be used to prepare compositions of the present invention.

Although the sequence of the variants proteins discussed above will differ from their wild-type counterparts, these variant proteins will generally retain the ability to form capsids or capsid-like structures. Thus, the invention further includes compositions and vaccine compositions, respectively, which further includes variants of proteins which form capsids or capsid-like structures, as well as methods for preparing such compositions and vaccine compositions, respectively, individual protein subunits used to prepare such



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compositions, and nucleic acid molecules which encode these protein subunits. Thus, included within the scope of the invention are variant forms of wild-type proteins which form capsids or capsid-like structures and retain the ability to associate and form capsids or capsid-like structures.

As a result, the invention further includes compositions and vaccine compositions, respectively, comprising proteins, which comprise, or alternatively consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to wild-type proteins which form ordered arrays and have an inherent repetitive structure, respectively.

Further included within the scope of the invention are nucleic acid molecules which encode proteins used to prepare compositions of the present invention.

In other embodiments, the invention further includes compositions comprising proteins, which comprise, or alternatively consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to any of the amino acid sequences shown in SEQ ID NOs:10-27.

Proteins suitable for use in the present invention also include C-terminal truncation mutants of proteins which form capsids or capsid-like structures, or VLPs. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:10-27 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Further proteins suitable for use in the present invention also include N-terminal truncation mutants of proteins which form capsids or capsid-like structures. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:10-27 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the

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N-terminus. Typically, these N-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Additional proteins suitable for use in the present invention include N- and C-terminal truncation mutants which form capsids or capsid-like structures. Suitable truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:10-27 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus and 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these N-terminal and C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

The invention further includes compositions comprising proteins which comprise, or alternatively consist essentially of, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

The invention thus includes compositions and vaccine compositions prepared from proteins which form capsids or VLPs, methods for preparing these compositions from individual protein subunits and VLPs or capsids, methods for preparing these individual protein subunits, nucleic acid molecules which encode these subunits, and methods for vaccinating and/or eliciting immunological responses in individuals using these compositions of the present invention.

Fragments of VLPs which retain the ability to induce an immune response can comprise, or alternatively consist of, polypeptides which are about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450 or 500 amino acids in length, but will obviously depend on the length of the sequence of the subunit composing the VLP. Examples of such fragments include fragments of proteins discussed herein which are suitable for the preparation of the immune response enhancing composition.

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In another preferred embodiment of the invention, the VLP's are free of a lipoprotein envelope or a lipoprotein-containing envelope. In a further preferred embodiment, the VLP's are free of an envelope altogether.

The lack of a lipoprotein envelope or lipoprotein-containing envelope and, in particular, the complete lack of an envelope leads to a more defined virus-like particle in its structure and composition. Such more defined virus-like particles, therefore, may minimize side-effects. Moreover, the lack of a lipoprotein-containing envelope or, in particular, the complete lack of an envelope avoids or minimizes incorporation of potentially toxic molecules and pyrogens within the virus-like particle.

As previously stated, the invention includes virus-like particles or recombinant forms thereof. Skilled artisans have the knowledge to produce such particles and attach antigens thereto. By way of providing other examples, the invention provides herein for the production of Hepatitis B virus-like particles as virus-like particles (Example 1).

In one embodiment, the particles used in compositions of the invention are composed of a Hepatitis B capsid (core) protein (HBcAg) or a fragment of a HBcAg which has been modified to either eliminate or reduce the number of free cysteine residues. Zhou *et al.* (*J. Virol.* 66:5393-5398 (1992)) demonstrated that HBcAgs which have been modified to remove the naturally resident cysteine residues retain the ability to associate and form multimeric structures. Thus, core particles suitable for use in compositions of the invention include those comprising modified HBcAgs, or fragments thereof, in which one or more of the naturally resident cysteine residues have been either deleted or substituted with another amino acid residue (*e.g.*, a serine residue).

The HBcAg is a protein generated by the processing of a Hepatitis B core antigen precursor protein. A number of isotypes of the HBcAg have been identified and their amino acids sequences are readily available to those skilled in the art. For example, the HBcAg protein having the amino acid sequence shown in Figure 1 is 185 amino acids in length and is generated by the processing of a 212 amino acid Hepatitis B core antigen precursor protein.

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This processing results in the removal of 29 amino acids from the N-terminus of the Hepatitis B core antigen precursor protein. Similarly, the HBcAg protein that is 185 amino acids in length is generated by the processing of a 214 amino acid Hepatitis B core antigen precursor protein.

In preferred embodiments, vaccine compositions of the invention will be prepared using the processed form of a HBcAg (*i.e.*, a HBcAg from which the N-terminal leader sequence of the Hepatitis B core antigen precursor protein have been removed).

Further, when HBcAgs are produced under conditions where processing will not occur, the HBcAgs will generally be expressed in "processed" form. For example, bacterial systems, such as *E. coli*, generally do not remove the leader sequences, also referred to as "signal peptides," of proteins which are normally expressed in eukaryotic cells. Thus, when an *E. coli* expression system directing expression of the protein to the cytoplasm is used to produce HBcAgs of the invention, these proteins will generally be expressed such that the N-terminal leader sequence of the Hepatitis B core antigen precursor protein is not present.

The preparation of Hepatitis B virus-like particles, which can be used for the present invention, is disclosed, for example, in WO 00/32227, and hereby in particular in Examples 17 to 19 and 21 to 24, as well as in WO 01/85208, and hereby in particular in Examples 17 to 19, 21 to 24, 31 and 41, and in pending U.S. Application No. 10/050,902 filed on January 18, 2002. For the latter application, it is in particular referred to Example 23, 24, 31 and 51. All three documents are explicitly incorporated herein by reference.

The present invention also includes HBcAg variants which have been modified to delete or substitute one or more additional cysteine residues. Thus, the vaccine compositions of the invention include compositions comprising HBcAgs in which cysteine residues not present in the amino acid sequence shown in Figure 1 have been deleted.

It is well known in the art that free cysteine residues can be involved in a number of chemical side reactions. These side reactions include disulfide

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exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy with other substances, or direct oxidation and reaction with nucleotides upon exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. The toxic adducts would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together.

In view of the above, one advantage to the use of HBcAgs in vaccine compositions which have been modified to remove naturally resident cysteine residues is that sites to which toxic species can bind when antigens or antigenic determinants are attached would be reduced in number or eliminated altogether.

A number of naturally occurring HBcAg variants suitable for use in the practice of the present invention have been identified. Yuan *et al.*, (*J. Virol.* 73:10122-10128 (1999)), for example, describe variants in which the isoleucine residue at position corresponding to position 97 in SEQ ID NO:28 is replaced with either a leucine residue or a phenylalanine residue. The amino acid sequences of a number of HBcAg variants, as well as several Hepatitis B core antigen precursor variants, are disclosed in GenBank reports AAF121240 (SEQ ID NO:29), AF121239 (SEQ ID NO:30), X85297 (SEQ ID NO:31), X02496 (SEQ ID NO:32), X85305 (SEQ ID NO:33), X85303 (SEQ ID NO:34), AF151735 (SEQ ID NO:35), X85259 (SEQ ID NO:36), X85286 (SEQ ID NO:37), X85260 (SEQ ID NO:38), X85317 (SEQ ID NO:39), X85298 (SEQ ID NO:40), AF043593 (SEQ ID NO:41), M20706 (SEQ ID NO:42), X85295 (SEQ ID NO:43), X80925 (SEQ ID NO:44), X85284 (SEQ ID NO:45), X85275 (SEQ ID NO:46), X72702 (SEQ ID NO:47), X85291 (SEQ ID NO:48), X65258 (SEQ ID NO:49), X85302 (SEQ ID NO:50), M32138 (SEQ ID NO:51), X85293 (SEQ ID NO:52), X85315 (SEQ ID NO:53), U95551 (SEQ ID NO:54), X85256 (SEQ ID NO:55), X85316 (SEQ ID NO:56), X85296 (SEQ ID NO:57), AB033559 (SEQ ID NO:58), X59795

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(SEQ ID NO:59), X85299 (SEQ ID NO:60), X85307 (SEQ ID NO:61), X65257 (SEQ ID NO:62), X85311 (SEQ ID NO:63), X85301 (SEQ ID NO:64), X85314 (SEQ ID NO:65), X85287 (SEQ ID NO:66), X85272 (SEQ ID NO:67), X85319 (SEQ ID NO:68), AB010289 (SEQ ID NO:69), X85285 (SEQ ID NO:70), AB010289 (SEQ ID NO:71), AF121242 (SEQ ID NO:72), M90520 (SEQ ID NO:73), P03153 (SEQ ID NO:74), AF110999 (SEQ ID NO:75), and M95589 (SEQ ID NO:76), the disclosures of each of which are incorporated herein by reference. These HBcAg variants differ in amino acid sequence at a number of positions, including amino acid residues which corresponds to the amino acid residues located at positions 12, 13, 21, 22, 24, 29, 32, 33, 35, 38, 40, 42, 44, 45, 49, 51, 57, 58, 59, 64, 66, 67, 69, 74, 77, 80, 81, 87, 92, 93, 97, 98, 100, 103, 105, 106, 109, 113, 116, 121, 126, 130, 133, 135, 141, 147, 149, 157, 176, 178, 182 and 183 in SEQ ID NO:77. Further HBcAg variants suitable for use in the compositions of the invention, and which may be further modified according to the disclosure of this specification are described in WO 00/198333, WO 00/177158 and WO 00/214478.

HBcAgs suitable for use in the present invention can be derived from any organism so long as they are able to enclose or to be coupled or otherwise attached to, in particular as long as they are capable of packaging, an unmethylated CpG-containing oligonucleotide and induce an immune response.

As noted above, generally processed HBcAgs (*i.e.*, those which lack leader sequences) will be used in the vaccine compositions of the invention. The present invention includes vaccine compositions, as well as methods for using these compositions, which employ the above described variant HBcAgs.

Further included within the scope of the invention are additional HBcAg variants which are capable of associating to form dimeric or multimeric structures. Thus, the invention further includes vaccine compositions comprising HBcAg polypeptides comprising, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms

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of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence.

Whether the amino acid sequence of a polypeptide has an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97% or 99% identical to one of the wild-type amino acid sequences, or a subportion thereof, can be determined conventionally using known computer programs such the Bestfit program. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference amino acid sequence, the parameters are set such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The HBcAg variants and precursors having the amino acid sequences set out in SEQ ID NOs: 29-72 and 73-76 are relatively similar to each other. Thus, reference to an amino acid residue of a HBcAg variant located at a position which corresponds to a particular position in SEQ ID NO:77, refers to the amino acid residue which is present at that position in the amino acid sequence shown in SEQ ID NO:77. The homology between these HBcAg variants is for the most part high enough among Hepatitis B viruses that infect mammals so that one skilled in the art would have little difficulty reviewing both the amino acid sequence shown in SEQ ID NO:77 and in Figure 1, respectively, and that of a particular HBcAg variant and identifying "corresponding" amino acid residues. Furthermore, the HBcAg amino acid sequence shown in SEQ ID NO:73, which shows the amino acid sequence of a HBcAg derived from a virus which infect woodchucks, has enough homology to the HBcAg having the amino acid sequence shown in SEQ ID NO:77 that it is readily apparent that a three amino acid residue insert is present in SEQ ID NO:73 between amino acid residues 155 and 156 of SEQ ID NO:77.

The invention also includes vaccine compositions which comprise HBcAg variants of Hepatitis B viruses which infect birds, as wells as vaccine compositions which comprise fragments of these HBcAg variants. As one

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skilled in the art would recognize, one, two, three or more of the cysteine residues naturally present in these polypeptides could be either substituted with another amino acid residue or deleted prior to their inclusion in vaccine compositions of the invention.

As discussed above, the elimination of free cysteine residues reduces the number of sites where toxic components can bind to the HBcAg, and also eliminates sites where cross-linking of lysine and cysteine residues of the same or of neighboring HBcAg molecules can occur. Therefore, in another embodiment of the present invention, one or more cysteine residues of the Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue.

In other embodiments, compositions and vaccine compositions, respectively, of the invention will contain HBcAgs from which the C-terminal region (*e.g.*, amino acid residues 145-185 or 150-185 of SEQ ID NO: 77) has been removed. Thus, additional modified HBcAgs suitable for use in the practice of the present invention include C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 5, 10, 15, 20, 25, 30, 34, 35, amino acids have been removed from the C-terminus.

HBcAgs suitable for use in the practice of the present invention also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus.

Further HBcAgs suitable for use in the practice of the present invention include N- and C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, 35 amino acids have been removed from the C-terminus.

The invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides comprising, or alternatively essentially consisting of, or alternatively consisting of, amino acid sequences



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which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

In certain embodiments of the invention, a lysine residue is introduced into a HBcAg polypeptide, to mediate the binding of the antigen or antigenic determinant to the VLP of HBcAg. In preferred embodiments, compositions of the invention are prepared using a HBcAg comprising, or alternatively consisting of, amino acids 1-144, or 1-149, 1-185 of SEQ ID NO:77, which is modified so that the amino acids corresponding to positions 79 and 80 are replaced with a peptide having the amino acid sequence of Gly-Gly-Lys-Gly-Gly (SEQ ID NO:78). These compositions are particularly useful in those embodiments where an antigenic determinant is coupled to a VLP of HBcAg. In further preferred embodiments, the cysteine residues at positions 48 and 107 of SEQ ID NO:77 are mutated to serine. The invention further includes compositions comprising the corresponding polypeptides having amino acid sequences shown in any of SEQ ID NOs:29-74 which also have above noted amino acid alterations. Further included within the scope of the invention are additional HBcAg variants which are capable of associating to form a capsid or VLP and have the above noted amino acid alterations. Thus, the invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides which comprise, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence and modified with above noted alterations.

Compositions or vaccine compositions of the invention may comprise mixtures of different HBcAgs. Thus, these vaccine compositions may be composed of HBcAgs which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (*e.g.*, deleted, inserted or substituted). Further, preferred vaccine

compositions of the invention are those which present highly ordered and repetitive antigen arrays.

As previously disclosed, the invention is based on the surprising finding that immunostimulatory substances, preferably immunostimulatory nucleic acids and even more preferably DNA oligonucleotides can be packaged into VLPs. Unexpectedly, the nucleic acids present in VLPs can be replaced specifically by the immunostimulatory substances, preferably by the immunostimulatory nucleic acids and even more preferably by the DNA-oligonucleotides containing CpG motifs. As an example, the CpG-VLPs are dramatically more immunogenic and elicit more specific effects than their CpG-free counterparts and induce enhanced B and T cell responses. The immune response against antigens coupled, fused or attached otherwise to the VLPs is similarly enhanced as the immune response against the VLP itself. In addition, the T cell responses against both the VLPs and antigens are especially directed to the Th1 type. Furthermore, the packaged nucleic acids and CpGs, respectively, are protected from degradation, *i.e.*, they are more stable. Moreover, non-specific activation of cells from the innate immune system is dramatically reduced.

The innate immune system has the capacity to recognize invariant molecular pattern shared by microbial pathogens. Recent studies have revealed that this recognition is a crucial step in inducing effective immune responses. The main mechanism by which microbial products augment immune responses is to stimulate APC, especially dendritic cells to produce proinflammatory cytokines and to express high levels costimulatory molecules for T cells. These activated dendritic cells subsequently initiate primary T cell responses and dictate the type of T cell-mediated effector function.

Two classes of nucleic acids, namely 1) bacterial DNA that contains immunostimulatory sequences, in particular unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) and 2) double-stranded RNA synthesized by various types of viruses represent important members of the microbial components that enhance immune responses.

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Synthetic double stranded (ds) RNA such as polyinosinic-polycytidylic acid (poly I:C) are capable of inducing dendritic cells to produce proinflammatory cytokines and to express high levels of costimulatory molecules.

A series of studies by Tokunaga and Yamamoto et al. has shown that bacterial DNA or synthetic oligodeoxynucleotides induce human PBMC and mouse spleen cells to produce type I interferon (IFN) (reviewed in Yamamoto et al., Springer Semin Immunopathol. 22:11-19). Poly (I:C) was originally synthesized as a potent inducer of type I IFN but also induces other cytokines such as IL-12.

Preferred ribonucleic acid encompass polyinosinic-polycytidylic acid double-stranded RNA (poly I:C). Ribonucleic acids and modifications thereof as well as methods for their production have been described by Levy, H.B (Methods Enzymol. 1981, 78:242-251), DeClercq, E (Methods Enzymol. 1981,78:227-236) and Torrence, P.F. (Methods Enzymol 1981;78:326-331) and references therein. Ribonucleic acids can be isolated from organisms. Ribonucleic acids also encompass further synthetic ribonucleic acids, in particular synthetic poly (I:C) oligonucleotides that have been rendered nuclease resistant by modification of the phosphodiester backbone, in particular by phosphorothioate modifications. In a further embodiment the ribose backbone of poly (I:C) is replaced by a deoxyribose. Those skilled in the art know procedures how to synthesize synthetic oligonucleotides.

In another preferred embodiment of the invention molecules that activate toll-like receptors (TLR) are enclosed. Ten human toll-like receptors are known uptodate. They are activated by a variety of ligands. TLR2 is activated by peptidoglycans, lipoproteins, lipoteichonic acid and Zymosan; TLR3 is activated by double-stranded RNA such as poly (I:C); TLR4 is activated by lipopolysaccharide, lipoteichoic acids and taxol; TLR5 is activated by bacterial flagella, especially the flagellin protein; TLR6 is activated by peptidoglycans, TLR7 is activated by imiquimoid and imidazoquinoline compounds, such as R818 and TLR9 is activated by bacterial DNA, in particular CpG DNA. Ligands for TLR1, TLR8 and TLR10 are not known so

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far. However, recent reports indicate that same receptors can react with different ligands and that further receptors are present. The above list of ligands is not exhaustive and further ligands are within the knowledge of the person skilled in the art.

Preferably, the unmethylated CpG-containing oligonucleotide comprises the sequence:



wherein  $X_1$ ,  $X_2$ ,  $X_3$  and  $X_4$  are any nucleotide. In addition, the oligonucleotide can comprise about 6 to about 100,000 nucleotides, preferably about 6 to about 2000 nucleotides, more preferably about 20 to about 2000 nucleotides, and even more preferably comprises about 20 to about 300 nucleotides. In addition, the oligonucleotide can comprise more than 100 to about 2000 nucleotides, preferably more than 100 to about 1000 nucleotides, and more preferably more than 100 to about 500 nucleotides.

In a preferred embodiment, the CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone. For example, a CpG-containing oligonucleotide having one or more phosphate backbone modifications or having all of the phosphate backbone modified and a CpG-containing oligonucleotide wherein one, some or all of the nucleotide phosphate backbone modifications are phosphorothioate modifications are included within the scope of the present invention.

The CpG-containing oligonucleotide can also be recombinant, genomic, synthetic, cDNA, plasmid-derived and single or double stranded. For use in the instant invention, the nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S. L., and Caruthers, M. H., *Tet. Let.* 22:1859 (1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054 (1986); Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407 (1986); Garegg *et al.*, *Tet. Let.* 27:4055-4058 (1986), Gaffney *et al.*, *Tet. Let.* 29:2619-2622 (1988)). These chemistries can be performed by a variety of

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automated oligonucleotide synthesizers available in the market. Alternatively, CpGs can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides. Oligonucleotides can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

The immunostimulatory substances, the immunostimulatory nucleic acids as well as the unmethylated CpG-containing oligonucleotide can be bound to the VLP by any way known in the art provided the composition enhances an immune response in an animal. For example, the oligonucleotide can be bound either covalently or non-covalently. In addition, the VLP can enclose, fully or partially, the immunostimulatory substances, the immunostimulatory nucleic acids as well as the unmethylated CpG-containing oligonucleotide. Preferably, the immunostimulatory nucleic acid as well as the unmethylated CpG-containing oligonucleotide can be bound to a VLP site such as an oligonucleotide binding site (either naturally or non-naturally occurring), a DNA binding site or a RNA binding site. In another embodiment, the VLP site comprises an arginine-rich repeat.

One specific use for the compositions of the invention is to activate dendritic cells for the purpose of enhancing a specific immune response against antigens. The immune response can be enhanced using ex vivo or in vivo techniques. The ex vivo procedure can be used on autologous or heterologous cells, but is preferably used on autologous cells. In preferred embodiments, the dendritic cells are isolated from peripheral blood or bone marrow, but can be isolated from any source of dendritic cells. Ex vivo manipulation of dendritic cells for the purposes of cancer immunotherapy have been described in several references in the art, including Engleman, E. G., *Cytotechnology* 25:1 (1997); Van Schooten, W., *et al.*, *Molecular Medicine Today*, June, 255 (1997); Steinman, R. M., *Experimental Hematology* 24:849

(1996); and Gluckman, J. C., *Cytokines, Cellular and Molecular Therapy* 3:187 (1997).

The dendritic cells can also be contacted with the inventive compositions using in vivo methods. In order to accomplish this, the CpGs are administered in combination with the VLP optionally coupled, fused or otherwise attached to an antigen directly to a subject in need of immunotherapy. In some embodiments, it is preferred that the VLPs/CpGs be administered in the local region of the tumor, which can be accomplished in any way known in the art, e.g., direct injection into the tumor.

The inventive composition can further comprise an antigen or antigenic determinant bound to the virus-like particle. The invention provides for compositions that vary according to the antigen or antigenic determinant selected in consideration of the desired therapeutic effect. Very preferred antigens or antigenic determinants suitable for use in the present invention are disclosed in WO 00/32227, in WO 01/85208 and in WO 02/056905, the disclosures of which are herewith incorporated by reference in their entireties.

The antigen can be any antigen of known or yet unknown provenance. It can be isolated from bacteria, viruses or other pathogens or can be a recombinant antigen obtained from expression of suitable nucleic acid coding therefor. It can also be isolated from prions, tumors, self-molecules, non-peptidic hapten molecules, allergens and hormones. In a preferred embodiment, the antigen is a recombinant antigen. The selection of the antigen is, of course, dependent upon the immunological response desired and the host.

In one embodiment of the immune enhancing composition of the present invention, the immune response is induced against the VLP itself. In another embodiment of the invention a virus-like particle is coupled, fused or otherwise attached to an antigen/immunogen against which an enhanced immune response is desired.

In a further preferred embodiment of the invention, the at least one antigen or antigenic determinant is fused to the virus-like particle. As outlined

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above, a VLP is typically composed of at least one subunit assembling into a VLP. Thus, in again a further preferred embodiment of the invention, the antigen or antigenic determinant is fused to at least one subunit of the virus-like particle or of a protein capable of being incorporated into a VLP generating a chimeric VLP-subunit-antigen fusion.

Fusion of the antigen or antigenic determinant can be effected by insertion into the VLP subunit sequence, or by fusion to either the N- or C-terminus of the VLP-subunit or protein capable of being incorporated into a VLP. Hereinafter, when referring to fusion proteins of a peptide to a VLP subunit, the fusion to either ends of the subunit sequence or internal insertion of the peptide within the subunit sequence are encompassed.

Fusion may also be effected by inserting antigen or antigenic determinant sequences into a variant of a VLP subunit where part of the subunit sequence has been deleted, that are further referred to as truncation mutants. Truncation mutants may have N- or C-terminal, or internal deletions of part of the sequence of the VLP subunit. For example, the specific VLP HBcAg with, for example, deletion of amino acid residues 79 to 81 is a truncation mutant with an internal deletion. Fusion of antigens or antigenic determinants to either the N- or C-terminus of the truncation mutants VLP-subunits also lead to embodiments of the invention. Likewise, fusion of an epitope into the sequence of the VLP subunit may also be effected by substitution, where for example for the specific VLP HBcAg, amino acids 79-81 are replaced with a foreign epitope. Thus, fusion, as referred to hereinafter, may be effected by insertion of the antigen or antigenic determinant sequence in the sequence of a VLP subunit, by substitution of part of the sequence of the VLP subunit with the antigen or antigenic determinant, or by a combination of deletion, substitution or insertions.

The chimeric antigen or antigenic determinant -VLP subunit will be in general capable of self-assembly into a VLP. VLP displaying epitopes fused to their subunits are also herein referred to as chimeric VLPs. As indicated, the virus-like particle comprises or alternatively is composed of at least one VLP

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subunit. In a further embodiment of the invention, the virus-like particle comprises or alternatively is composed of a mixture of chimeric VLP subunits and non-chimeric VLP subunits, i.e. VLP subunits not having an antigen fused thereto, leading to so called mosaic particles. This may be advantageous to ensure formation of, and assembly to a VLP. In those embodiments, the proportion of chimeric VLP-subunits may be 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95% or higher.

Flanking amino acid residues may be added to either end of the sequence of the peptide or epitope to be fused to either end of the sequence of the subunit of a VLP, or for internal insertion of such peptidic sequence into the sequence of the subunit of a VLP. Glycine and serine residues are particularly favored amino acids to be used in the flanking sequences added to the peptide to be fused. Glycine residues confer additional flexibility, which may diminish the potentially destabilizing effect of fusing a foreign sequence into the sequence of a VLP subunit.

In a specific embodiment of the invention, the VLP is a Hepatitis B core antigen VLP. Fusion proteins of the antigen or antigenic determinant to either the N-terminus of a HBcAg (Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) or insertions in the so called major immunodominant region (MIR) have been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)), WO 01/98333), and are preferred embodiments of the invention. Naturally occurring variants of HBcAg with deletions in the MIR have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001), which is expressly incorporated by reference in its entirety), and fusions to the N- or C-terminus, as well as insertions at the position of the MIR corresponding to the site of deletion as compared to a wt HBcAg are further embodiments of the invention. Fusions to the C-terminus have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). One skilled in the art will easily find guidance on how to construct fusion proteins using classical molecular biology techniques (Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Cold Spring Harbor



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Laboratory Press, Cold Spring Harbor, N.Y. (1989), Ho *et al.*, *Gene* 77:51 (1989)). Vectors and plasmids encoding HBcAg and HBcAg fusion proteins and useful for the expression of a HBcAg and HBcAg fusion proteins have been described (Pumpens, P. & Grens, E. *Intervirology* 44: 98-114 (2001), Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) and can be used in the practice of the invention. An important factor for the optimization of the efficiency of self-assembly and of the display of the epitope to be inserted in the MIR of HBcAg is the choice of the insertion site, as well as the number of amino acids to be deleted from the HBcAg sequence within the MIR (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001); EP 0 421 635; U.S. Patent No. 6,231,864) upon insertion, or in other words, which amino acids form HBcAg are to be substituted with the new epitope. For example, substitution of HBcAg amino acids 76-80, 79-81, 79-80, 75-85 or 80-81 with foreign epitopes has been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001); EP0421635; US 6,231,864). HBcAg contains a long arginine tail (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)) which is dispensable for capsid assembly and capable of binding nucleic acids (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). HBcAg either comprising or lacking this arginine tail are both embodiments of the invention.

In a further preferred embodiment of the invention, the VLP is a VLP of a RNA phage. The major coat proteins of RNA phages spontaneously assemble into VLPs upon expression in bacteria, and in particular in *E. coli*. Specific examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA bacteriophages such as bacteriophage Q $\beta$  (SEQ ID NO:10; PIR Database, Accession No. VCBPQ $\beta$  referring to Q $\beta$  CP and SEQ ID NO: 11; Accession No. AAA16663 referring to Q $\beta$  A1 protein) and bacteriophage fr (SEQ ID NO: 13; PIR Accession No. VCBPFR).

In a more preferred embodiment, the at least one antigen or antigenic determinant is fused to a Q $\beta$  coat protein. Fusion protein constructs wherein

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epitopes have been fused to the C-terminus of a truncated form of the A1 protein of Q $\beta$ , or inserted within the A1 protein have been described (Kozlovskaya, T. M., *et al.*, *Intervirology*, 39:9-15 (1996)). The A1 protein is generated by suppression at the UGA stop codon and has a length of 329 aa, or 328 aa, if the cleavage of the N-terminal methionine is taken into account. Cleavage of the N-terminal methionine before an alanine (the second amino acid encoded by the Q $\beta$  CP gene) usually takes place in *E. coli*, and such is the case for N-termini of the Q $\beta$  coat proteins. The part of the A1 gene, 3' of the UGA amber codon encodes the CP extension, which has a length of 195 amino acids. Insertion of the at least one antigen or antigenic determinant between position 72 and 73 of the CP extension leads to further embodiments of the invention (Kozlovskaya, T. M., *et al.*, *Intervirology* 39:9-15 (1996)). Fusion of an antigen or antigenic determinant at the C-terminus of a C-terminally truncated Q $\beta$  A1 protein leads to further preferred embodiments of the invention. For example, Kozlovskaya *et al.*, (*Intervirology*, 39: 9-15 (1996)) describe Q $\beta$  A1 protein fusions where the epitope is fused at the C-terminus of the Q $\beta$  CP extension truncated at position 19.

As described by Kozlovskaya *et al.* (*Intervirology*, 39: 9-15 (1996)), assembly of the particles displaying the fused epitopes typically requires the presence of both the A1 protein-antigen fusion and the wt CP to form a mosaic particle. However, embodiments comprising virus-like particles, and hereby in particular the VLPs of the RNA phage Q $\beta$  coat protein, which are exclusively composed of VLP subunits having at least one antigen or antigenic determinant fused thereto, are also within the scope of the present invention.

The production of mosaic particles may be effected in a number of ways. Kozlovskaya *et al.*, *Intervirology*, 39:9-15 (1996), describe three methods, which all can be used in the practice of the invention. In the first approach, efficient display of the fused epitope on the VLPs is mediated by the expression of the plasmid encoding the Q $\beta$  A1 protein fusion having a UGA stop codon between CP and CP extension in a *E. coli* strain harboring a

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plasmid encoding a cloned UGA suppressor tRNA which leads to translation of the UGA codon into Trp (pISM3001 plasmid (Smiley B.K., *et al.*, *Gene* 134:33-40 (1993))). In another approach, the CP gene stop codon is modified into UAA, and a second plasmid expressing the A1 protein-antigen fusion is cotransformed. The second plasmid encodes a different antibiotic resistance and the origin of replication is compatible with the first plasmid (Kozlovskaya, T. M., *et al.*, *Intervirology* 39:9-15 (1996)). In a third approach, CP and the A1 protein-antigen fusion are encoded in a bicistronic manner, operatively linked to a promoter such as the Trp promoter, as described in Figure 1 of Kozlovskaya *et al.*, *Intervirology*, 39:9-15 (1996).

In a further embodiment, the antigen or antigenic determinant is inserted between amino acid 2 and 3 (numbering of the cleaved CP, that is wherein the N-terminal methionine is cleaved) of the fr CP, thus leading to an antigen or antigenic determinant -fr CP fusion protein. Vectors and expression systems for construction and expression of fr CP fusion proteins self-assembling to VLP and useful in the practice of the invention have been described (Pushko P. *et al.*, *Prot. Eng.* 6:883-891 (1993)). In a specific embodiment, the antigen or antigenic determinant sequence is inserted into a deletion variant of the fr CP after amino acid 2, wherein residues 3 and 4 of the fr CP have been deleted (Pushko P. *et al.*, *Prot. Eng.* 6:883-891 (1993)).

Fusion of epitopes in the N-terminal protuberant  $\beta$ -hairpin of the coat protein of RNA phage MS-2 and subsequent presentation of the fused epitope on the self-assembled VLP of RNA phage MS-2 has also been described (WO 92/13081), and fusion of an antigen or antigenic determinant by insertion or substitution into the coat protein of MS-2 RNA phage is also falling under the scope of the invention.

In another embodiment of the invention, the antigen or antigenic determinant is fused to a capsid protein of papillomavirus. In a more specific embodiment, the antigen or antigenic determinant is fused to the major capsid protein L1 of bovine papillomavirus type 1 (BPV-1). Vectors and expression systems for construction and expression of BPV-1 fusion proteins in a

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baculovirus/insect cells systems have been described (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Substitution of amino acids 130-136 of BPV-1 L1 with an antigen or antigenic determinant leads to a BPV-1 L1-antigen fusion protein, which is a preferred embodiment of the invention. Cloning in a baculovirus vector and expression in baculovirus infected Sf9 cells has been described, and can be used in the practice of the invention (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Purification of the assembled particles displaying the fused antigen or antigenic determinant can be performed in a number of ways, such as for example gel filtration or sucrose gradient ultracentrifugation (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955).

In a further embodiment of the invention, the antigen or antigenic determinant is fused to a Ty protein capable of being incorporated into a Ty VLP. In a more specific embodiment, the antigen or antigenic determinant is fused to the p1 or capsid protein encoded by the TYA gene (Roth, J.F., *Yeast* 16:785-795 (2000)). The yeast retrotransposons Ty1, 2, 3 and 4 have been isolated from *Saccharomyces Serevisiae*, while the retrotransposon Tfl has been isolated from *Schizosaccharomyces Pombae* (Boeke, J.D. and Sandmeyer, S.B., "Yeast Transposable elements," in *The molecular and Cellular Biology of the Yeast Saccharomyces: Genome dynamics, Protein Synthesis, and Energetics*, p. 193, Cold Spring Harbor Laboratory Press (1991)). The retrotransposons Ty1 and 2 are related to the *copia* class of plant and animal elements, while Ty3 belongs to the *gypsy* family of retrotransposons, which is related to plants and animal retroviruses. In the Ty1 retrotransposon, the p1 protein, also referred to as Gag or capsid protein, has a length of 440 amino acids. P1 is cleaved during maturation of the VLP at position 408, leading to the p2 protein, the essential component of the VLP.

Fusion proteins to p1 and vectors for the expression of said fusion proteins in Yeast have been described (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). So, for example, an antigen or antigenic determinant may be fused to

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p1 by inserting a sequence coding for the antigen or antigenic determinant into the BamHI site of the pMA5620 plasmid (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). The cloning of sequences coding for foreign epitopes into the pMA5620 vector leads to expression of fusion proteins comprising amino acids 1-381 of p1 of Ty1-15, fused C-terminally to the N-terminus of the foreign epitope. Likewise, N-terminal fusion of an antigen or antigenic determinant, or internal insertion into the p1 sequence, or substitution of part of the p1 sequence are also meant to fall within the scope of the invention. In particular, insertion of an antigen or antigenic determinant into the Ty sequence between amino acids 30-31, 67-68, 113-114 and 132-133 of the Ty protein p1 (EP0677111) leads to preferred embodiments of the invention.

Further VLPs suitable for fusion of antigens or antigenic determinants are, for example, Retrovirus-like-particles (WO9630523), HIV2 Gag (Kang, Y.C., *et al.*, *Biol. Chem.* 380:353-364 (1999)), Cowpea Mosaic Virus (Taylor, K.M.*et al.*, *Biol. Chem.* 380:387-392 (1999)), parvovirus VP2 VLP (Rueda, P. *et al.*, *Virology* 263:89-99 (1999)), HBsAg (US 4,722,840, EP0020416B1).

Examples of chimeric VLPs suitable for the practice of the invention are also those described in *Intervirology* 39:1 (1996). Further examples of VLPs contemplated for use in the invention are: HPV-1, HPV-6, HPV-11, HPV-16, HPV-18, HPV-33, HPV-45, CRPV, COPV, HIV GAG, Tobacco Mosaic Virus. Virus-like particles of SV-40, Polyomavirus, Adenovirus, Herpes Simplex Virus, Rotavirus and Norwalk virus have also been made, and chimeric VLPs of those VLPs comprising an antigen or antigenic determinant are also within the scope of the present invention.

As indicated, embodiments comprising antigens fused to the virus-like particle by insertion within the sequence of the virus-like particle building monomer are also within the scope of the present invention. In some cases, antigens can be inserted in a form of the virus-like particle building monomer containing deletions. In these cases, the virus-like particle building monomer may not be able to form virus-like structures in the absence of the inserted antigen.

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In some instances, recombinant DNA technology can be utilized to fuse a heterologous protein to a VLP protein (Kratz, P.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 96:1915 (1999)). For example, the present invention encompasses VLPs recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to an antigen (or portion thereof, preferably at least 10, 20 or 50 amino acids) of the present invention to generate fusion proteins or conjugates. The fusion does not necessarily need to be direct, but can occur through linker sequences. More generally, in the case that epitopes, either fused, conjugated or otherwise attached to the virus-like particle, are used as antigens in accordance with the invention, spacer or linker sequences are typically added at one or both ends of the epitopes. Such linker sequences preferably comprise sequences recognized by the proteasome, proteases of the endosomes or other vesicular compartment of the cell.

One way of coupling is by a peptide bond, in which the conjugate can be a contiguous polypeptide, *i.e.* a fusion protein. In a fusion protein according to the present invention, different peptides or polypeptides are linked in frame to each other to form a contiguous polypeptide. Thus a first portion of the fusion protein comprises an antigen or immunogen and a second portion of the fusion protein, either N-terminal or C-terminal to the first portion, comprises a VLP. Alternatively, internal insertion into the VLP, with optional linking sequences on both ends of the antigen, can also be used in accordance with the present invention.

When HBcAg is used as the VLP, it is preferred that the antigen is linked to the C-terminal end of the HBcAg particle. The hepatitis B core antigen (HBcAg) exhibiting a C-terminal fusion of the MHC class I restricted peptide p33 derived from lymphocytic choriomeningitis virus (LCMV) glycoprotein was used as a model antigen (HBcAg-p33). The 185 amino acids long wild type HBc protein assembles into highly structured particles composed of 180 subunits assuming icosahedral geometry. The flexibility of the HBcAg and other VLPs in accepting relatively large insertions of foreign

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sequences at different positions while retaining the capacity to form structured capsids is well documented in the literature. This makes the HBc VLPs attractive candidates for the design of non-replicating vaccines.

A flexible linker sequence (e.g. a polyglycine/polyserine-containing sequence such as  $[\text{Gly}_4 \text{Ser}]_2$  (Huston *et al.*, *Meth. Enzymol* 203:46-88 (1991)) can be inserted into the fusion protein between the antigen and ligand. Also, the fusion protein can be constructed to contain an "epitope tag", which allows the fusion protein to bind an antibody (e.g. monoclonal antibody) for example for labeling or purification purposes. An example of an epitope tag is a Glu-Glu-Phe tripeptide which is recognized by the monoclonal antibody YL1/2.

The invention also relates to the chimeric DNA which contains a sequence coding for the VLP and a sequence coding for the antigen/immunogen. The DNA can be expressed, for example, in insect cells transformed with Baculoviruses, in yeast or in bacteria. There are no restrictions regarding the expression system, of which a large selection is available for routine use. Preferably, a system is used which allows expression of the proteins in large amounts. In general, bacterial expression systems are preferred on account of their efficiency. One example of a bacterial expression system suitable for use within the scope of the present invention is the one described by Clarke *et al.*, *J. Gen. Virol.* 71: 1109-1117 (1990); Borisova *et al.*, *J. Virol.* 67: 3696-3701 (1993); and Studier *et al.*, *Methods Enzymol.* 185:60-89 (1990). An example of a suitable yeast expression system is the one described by Emr, *Methods Enzymol.* 185:231-3 (1990); Baculovirus systems, which have previously been used for preparing capsid proteins, are also suitable. Constitutive or inducible expression systems can be used. By the choice and possible modification of available expression systems it is possible to control the form in which the proteins are obtained.

In a specific embodiment of the invention, the antigen to which an enhanced immune response is desired is coupled, fused or otherwise attached in frame to the Hepatitis B virus capsid (core) protein (HBcAg). However, it

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will be clear to all individuals in the art that other virus-like particles can be utilized in the fusion protein construct of the invention.

In a further preferred embodiment of the present invention, the at least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond. Preferably, the least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond, said covalent bond being a non-peptide bond leading to an antigen or antigenic determinant array and antigen or antigenic determinant -VLP conjugate, respectively. This antigen or antigenic determinant array and conjugate, respectively, has typically and preferably a repetitive and ordered structure since the at least one antigen or antigenic determinant is bound to the VLP in an oriented manner. The formation of a repetitive and ordered antigen or antigenic determinant -VLP array and conjugate, respectively, is ensured by an oriented and directed as well as defined binding and attachment, respectively, of the at least one antigen or antigenic determinant to the VLP as will become apparent in the following. Furthermore, the typical inherent highly repetitive and organized structure of the VLPs advantageously contributes to the display of the antigen or antigenic determinant in a highly ordered and repetitive fashion leading to a highly organized and repetitive antigen or antigenic determinant -VLP array and conjugate, respectively.

Therefore, the preferred inventive conjugates and arrays, respectively, differ from prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. The preferred embodiment of this invention, furthermore, allows expression of the particle in an expression host guaranteeing proper folding and assembly of the VLP, to which the antigen is then further coupled

The present invention discloses methods of binding of antigen or antigenic determinant to VLPs. As indicated, in one aspect of the invention, the at least one antigen or antigenic determinant is bound to the VLP by way of chemical cross-linking, typically and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are



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known to the art. In preferred embodiments, the hetero-bifunctional cross-linker contains a functional group which can react with preferred first attachment sites, i.e. with the side-chain amino group of lysine residues of the VLP or at least one VLP subunit, and a further functional group which can react with a preferred second attachment site, i.e. a cysteine residue fused to the antigen or antigenic determinant and optionally also made available for reaction by reduction. The first step of the procedure, typically called the derivatization, is the reaction of the VLP with the cross-linker. The product of this reaction is an activated VLP, also called activated carrier. In the second step, unreacted cross-linker is removed using usual methods such as gel filtration or dialysis. In the third step, the antigen or antigenic determinant is reacted with the activated VLP, and this step is typically called the coupling step. Unreacted antigen or antigenic determinant may be optionally removed in a fourth step, for example by dialysis. Several hetero-bifunctional cross-linkers are known to the art. These include the preferred cross-linkers SMPH (Pierce), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example from the Pierce Chemical Company (Rockford, IL, USA), and having one functional group reactive towards amino groups and one functional group reactive towards cysteine residues. The above mentioned cross-linkers all lead to formation of a thioether linkage. Another class of cross-linkers suitable in the practice of the invention is characterized by the introduction of a disulfide linkage between the antigen or antigenic determinant and the VLP upon coupling. Preferred cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce). The extent of derivatization of the VLP with cross-linker can be influenced by varying experimental conditions such as the concentration of each of the reaction partners, the excess of one reagent over the other, the pH, the temperature and the ionic strength. The degree of coupling, i.e. the amount of antigens or antigenic determinants per subunits of the VLP can be adjusted by varying the experimental conditions described above to match the requirements of the vaccine.

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A particularly favored method of binding of antigens or antigenic determinants to the VLP, is the linking of a lysine residue on the surface of the VLP with a cysteine residue on the antigen or antigenic determinant. In some embodiments, fusion of an amino acid linker containing a cysteine residue, as a second attachment site or as a part thereof, to the antigen or antigenic determinant for coupling to the VLP may be required.

In general, flexible amino acid linkers are favored. Examples of the amino acid linker are selected from the group consisting of: (a) CGG; (b) N-terminal gamma 1-linker; (c) N-terminal gamma 3-linker; (d) Ig hinge regions; (e) N-terminal glycine linkers; (f)  $(G)_kC(G)_n$  with  $n=0-12$  and  $k=0-5$ ; (g) N-terminal glycine-serine linkers; (h)  $(G)_kC(G)_m(S)_l(GGGGS)_n$  with  $n=0-3$ ,  $k=0-5$ ,  $m=0-10$ ,  $l=0-2$ ; (i) GGC; (k) GGC-NH<sub>2</sub>; (l) C-terminal gamma 1-linker; (m) C-terminal gamma 3-linker; (n) C-terminal glycine linkers; (o)  $(G)_nC(G)_k$  with  $n=0-12$  and  $k=0-5$ ; (p) C-terminal glycine-serine linkers; (q)  $(G)_m(S)_l(GGGGS)_n(G)_oC(G)_k$  with  $n=0-3$ ,  $k=0-5$ ,  $m=0-10$ ,  $l=0-2$ , and  $o=0-8$ .

Further examples of amino acid linkers are the hinge region of Immunoglobulins, glycine serine linkers  $(GGGGS)_n$ , and glycine linkers  $(G)_n$  all further containing a cysteine residue as second attachment site and optionally further glycine residues. Typically preferred examples of said amino acid linkers are N-terminal gamma 1: CGDKTHTSPP; C-terminal gamma 1: DKTHTSPPCG; N-terminal gamma 3: CGGPKPSTPPGSSGGAP; C-terminal gamma 3: PKPSTPPGSSGGAPGGCG; N-terminal glycine linker: GCGGGG and C-terminal glycine linker: GGGGCG.

Other amino acid linkers particularly suitable in the practice of the invention, when a hydrophobic antigen or antigenic determinant is bound to a VLP, are CGKKGG, or CGDEGG for N-terminal linkers, or GGKKGC and GGEDGC, for the C-terminal linkers. For the C-terminal linkers, the terminal cysteine is optionally C-terminally amidated.

In preferred embodiments of the present invention, GGCG, GGC or GGC-NH<sub>2</sub> ("NH<sub>2</sub>" stands for amidation) linkers at the C-terminus of the peptide or CGG at its N-terminus are preferred as amino acid linkers. In

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general, glycine residues will be inserted between bulky amino acids and the cysteine to be used as second attachment site, to avoid potential steric hindrance of the bulkier amino acid in the coupling reaction. In the most preferred embodiment of the invention, the amino acid linker GGC-NH<sub>2</sub> is fused to the C-terminus of the antigen or antigenic determinant.

The cysteine residue present on the antigen or antigenic determinant has to be in its reduced state to react with the hetero-bifunctional cross-linker on the activated VLP, that is a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instance where the cysteine residue to function as binding site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with *e.g.* DTT, TCEP or  $\beta$ -mercaptoethanol is required. Low concentrations of reducing agent are compatible with coupling as described in WO 02/05690, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductant has to be removed or its concentration decreased prior to coupling, *e.g.* by dialysis, gel filtration or reverse phase HPLC.

Binding of the antigen or antigenic determinant to the VLP by using a hetero-bifunctional cross-linker according to the preferred methods described above, allows coupling of the antigen or antigenic determinant to the VLP in an oriented fashion. Other methods of binding the antigen or antigenic determinant to the VLP include methods wherein the antigen or antigenic determinant is cross-linked to the VLP using the carbodiimide EDC, and NHS. In further methods, the antigen or antigenic determinant is attached to the VLP using a homo-bifunctional cross-linker such as glutaraldehyde, DSG, BM[PEO]<sub>4</sub>, BS<sup>3</sup>, (Pierce Chemical Company, Rockford, IL, USA) or other known homo-bifunctional cross-linkers which functional groups reactive towards amine groups or carboxyl groups of the VLP.

Other methods of binding the VLP to an antigen or antigenic determinant include methods where the VLP is biotinylated, and the antigen or antigenic determinant expressed as a streptavidin-fusion protein, or methods wherein both the antigen or antigenic determinant and the VLP are

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biotinylated, for example as described in WO 00/23955. In this case, the antigen or antigenic determinant may be first bound to streptavidin or avidin by adjusting the ratio of antigen or antigenic determinant to streptavidin such that free binding sites are still available for binding of the VLP, which is added in the next step. Alternatively, all components may be mixed in a "one pot" reaction. Other ligand-receptor pairs, where a soluble form of the receptor and of the ligand is available, and are capable of being cross-linked to the VLP or the antigen or antigenic determinant, may be used as binding agents for binding antigen or antigenic determinant to the VLP. Alternatively, either the ligand or the receptor may be fused to the antigen or antigenic determinant, and so mediate binding to the VLP chemically bound or fused either to the receptor, or the ligand respectively. Fusion may also be effected by insertion or substitution.

As already indicated, in a favored embodiment of the present invention, the VLP is the VLP of a RNA phage, and in a more preferred embodiment, the VLP is the VLP of RNA phage Q $\beta$  coat protein.

One or several antigen molecules, i.e. one or several antigens or antigenic determinants, can be attached to one subunit of the capsid or VLP of RNA phages coat proteins, preferably through the exposed lysine residues of the VLP of RNA phages, if sterically allowable. A specific feature of the VLP of the coat protein of RNA phages and in particular of the Q $\beta$  coat protein VLP is thus the possibility to couple several antigens per subunit. This allows for the generation of a dense antigen array.

In a preferred embodiment of the invention, the binding and attachment, respectively, of the at least one antigen or antigenic determinant to the virus-like particle is by way of interaction and association, respectively, between at least one first attachment site of the virus-like particle and at least one second attachment of the antigen or antigenic determinant.

VLPs or capsids of Q $\beta$  coat protein display a defined number of lysine residues on their surface, with a defined topology with three lysine residues pointing towards the interior of the capsid and interacting with the RNA, and

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four other lysine residues exposed to the exterior of the capsid. These defined properties favor the attachment of antigens to the exterior of the particle, rather than to the interior of the particle where the lysine residues interact with RNA. VLPs of other RNA phage coat proteins also have a defined number of lysine residues on their surface and a defined topology of these lysine residues.

In further preferred embodiments of the present invention, the first attachment site is a lysine residue and/or the second attachment comprises a sulfhydryl group or a cysteine residue. In a very preferred embodiment of the present invention, the first attachment site is a lysine residue and the second attachment is a cysteine residue.

In very preferred embodiments of the invention, the antigen or antigenic determinant is bound via a cysteine residue, to lysine residues of the VLP of RNA phage coat protein, and in particular to the VLP of Q $\beta$  coat protein.

Another advantage of the VLPs derived from RNA phages is their high expression yield in bacteria that allows production of large quantities of material at affordable cost.

As indicated, the inventive conjugates and arrays, respectively, differ from prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. Moreover, the use of the VLPs as carriers allow the formation of robust antigen arrays and conjugates, respectively, with variable antigen density. In particular, the use of VLPs of RNA phages, and hereby in particular the use of the VLP of RNA phage Q $\beta$  coat protein allows to achieve very high epitope density. In particular, a density of more than 1.5 epitopes per subunit could be reached by coupling the human A $\beta$ 1-6 peptide to the VLP of Q $\beta$  coat protein. The preparation of compositions of VLPs of RNA phage coat proteins with a high epitope density can be effected using the teaching of this application. In preferred embodiment of the invention, when an antigen or antigenic determinant is coupled to the VLP of Q $\beta$  coat protein, an average number of

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antigen or antigenic determinant per subunit of 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or higher is preferred.

The second attachment site, as defined herein, may be either naturally or non-naturally present with the antigen or the antigenic determinant. In the case of the absence of a suitable natural occurring second attachment site on the antigen or antigenic determinant, such a, then non-natural second attachment has to be engineered to the antigen.

As described above, four lysine residues are exposed on the surface of the VLP of Q $\beta$  coat protein. Typically these residues are derivatized upon reaction with a cross-linker molecule. In the instance where not all of the exposed lysine residues can be coupled to an antigen, the lysine residues which have reacted with the cross-linker are left with a cross-linker molecule attached to the  $\epsilon$ -amino group after the derivatization step. This leads to disappearance of one or several positive charges, which may be detrimental to the solubility and stability of the VLP. By replacing some of the lysine residues with arginines, as in the disclosed Q $\beta$  coat protein mutants described below, we prevent the excessive disappearance of positive charges since the arginine residues do not react with the cross-linker. Moreover, replacement of lysine residues by arginines may lead to more defined antigen arrays, as fewer sites are available for reaction to the antigen.

Accordingly, exposed lysine residues were replaced by arginines in the following Q $\beta$  coat protein mutants and mutant Q $\beta$  VLPs disclosed in this application: Q $\beta$ -240 (Lys13-Arg; SEQ ID NO:23), Q $\beta$ -250 (Lys 2-Arg, Lys13-Arg; SEQ ID NO: 25) and Q $\beta$ -259 (Lys 2-Arg, Lys16-Arg; SEQ ID NO:27). The constructs were cloned, the proteins expressed, the VLPs purified and used for coupling to peptide and protein antigens. Q $\beta$ -251 ; (SEQ ID NO: 26) was also constructed, and guidance on how to express, purify and couple the VLP of Q $\beta$ -251 coat protein can be found throughout the application.

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In a further embodiment, we disclose a Q $\beta$  mutant coat protein with one additional lysine residue, suitable for obtaining even higher density arrays of antigens. This mutant Q $\beta$  coat protein, Q $\beta$ -243 (Asn 10-Lys; SEQ ID NO: 24), was cloned, the protein expressed, and the capsid or VLP isolated and purified, showing that introduction of the additional lysine residue is compatible with self-assembly of the subunits to a capsid or VLP. Thus, antigen or antigenic determinant arrays and conjugates, respectively, may be prepared using VLP of Q $\beta$  coat protein mutants. A particularly favored method of attachment of antigens to VLPs, and in particular to VLPs of RNA phage coat proteins is the linking of a lysine residue present on the surface of the VLP of RNA phage coat proteins with a cysteine residue added to the antigen. In order for a cysteine residue to be effective as second attachment site, a sulfhydryl group must be available for coupling. Thus, a cysteine residue has to be in its reduced state, that is, a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instant where the cysteine residue to function as second attachment site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with *e.g.* DTT, TCEP or  $\beta$ -mercaptoethanol is required. The concentration of reductant, and the molar excess of reductant over antigen has to be adjusted for each antigen. A titration range, starting from concentrations as low as 10  $\mu$ M or lower, up to 10 to 20 mM or higher reductant if required is tested, and coupling of the antigen to the carrier assessed. Although low concentrations of reductant are compatible with the coupling reaction as described in WO 02/056905, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductant has to be removed or its concentration decreased, *e.g.* by dialysis, gel filtration or reverse phase HPLC. Advantageously, the pH of the dialysis or equilibration buffer is lower than 7, preferably 6. The compatibility of the low pH buffer with antigen activity or stability has to be tested.

Epitope density on the VLP of RNA phage coat proteins can be modulated by the choice of cross-linker and other reaction conditions. For

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example, the cross-linkers Sulfo-GMBS and SMPH typically allow reaching high epitope density. Derivatization is positively influenced by high concentration of reactands, and manipulation of the reaction conditions can be used to control the number of antigens coupled to VLPs of RNA phage coat proteins, and in particular to VLPs of Q $\beta$  coat protein.

Prior to the design of a non-natural second attachment site the position at which it should be fused, inserted or generally engineered has to be chosen. The selection of the position of the second attachment site may, by way of example, be based on a crystal structure of the antigen. Such a crystal structure of the antigen may provide information on the availability of the C- or N-termini of the molecule (determined for example from their accessibility to solvent), or on the exposure to solvent of residues suitable for use as second attachment sites, such as cysteine residues. Exposed disulfide bridges, as is the case for Fab fragments, may also be a source of a second attachment site, since they can be generally converted to single cysteine residues through mild reduction, with e.g. 2-mercaptoethylamine, TCEP,  $\beta$ -mercaptoethanol or DTT. Mild reduction conditions not affecting the immunogenicity of the antigen will be chosen. In general, in the case where immunization with a self-antigen is aiming at inhibiting the interaction of this self-antigen with its natural ligands, the second attachment site will be added such that it allows generation of antibodies against the site of interaction with the natural ligands. Thus, the location of the second attachment site will be selected such that steric hindrance from the second attachment site or any amino acid linker containing the same is avoided. In further embodiments, an antibody response directed at a site distinct from the interaction site of the self-antigen with its natural ligand is desired. In such embodiments, the second attachment site may be selected such that it prevents generation of antibodies against the interaction site of the self-antigen with its natural ligands.

Other criteria in selecting the position of the second attachment site include the oligomerization state of the antigen, the site of oligomerization, the presence of a cofactor, and the availability of experimental evidence



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disclosing sites in the antigen structure and sequence where modification of the antigen is compatible with the function of the self-antigen, or with the generation of antibodies recognizing the self-antigen.

In very preferred embodiments, the antigen or antigenic determinant comprises a single second attachment site or a single reactive attachment site capable of association with the first attachment sites on the core particle and the VLPs or VLP subunits, respectively. This further ensures a defined and uniform binding and association, respectively, of the at least one, but typically more than one, preferably more than 10, 20, 40, 80, 120 antigens to the core particle and VLP, respectively. The provision of a single second attachment site or a single reactive attachment site on the antigen, thus, ensures a single and uniform type of binding and association, respectively leading to a very highly ordered and repetitive array. For example, if the binding and association, respectively, is effected by way of a lysine- (as the first attachment site) and cysteine- (as a second attachment site) interaction, it is ensured, in accordance with this preferred embodiment of the invention, that only one cysteine residue per antigen, independent whether this cysteine residue is naturally or non-naturally present on the antigen, is capable of binding and associating, respectively, with the VLP and the first attachment site of the core particle, respectively. .

In some embodiments, engineering of a second attachment site onto the antigen require the fusion of an amino acid linker containing an amino acid suitable as second attachment site according to the disclosures of this invention. Therefore, in a preferred embodiment of the present invention, an amino acid linker is bound to the antigen or the antigenic determinant by way of at least one covalent bond. Preferably, the amino acid linker comprises, or alternatively consists of, the second attachment site. In a further preferred embodiment, the amino acid linker comprises a sulfhydryl group or a cysteine residue. In another preferred embodiment, the amino acid linker is cysteine. Some criteria of selection of the amino acid linker as well as further preferred

embodiments of the amino acid linker according to the invention have already been mentioned above.

In another specific embodiment of the invention, the attachment site is selected to be a lysine or cysteine residue that is fused in frame to the HBcAg. In a preferred embodiment, the antigen is fused to the C-terminus of HBcAg via a three leucine linker.

When an antigen or antigenic determinant is linked to the VLP through a lysine residue, it may be advantageous to either substitute or delete one or more of the naturally resident lysine residues, as well as other lysine residues present in HBcAg variants.

In many instances, when the naturally resident lysine residues are eliminated, another lysine will be introduced into the HBcAg as an attachment site for an antigen or antigenic determinant. Methods for inserting such a lysine residue are known in the art. Lysine residues may also be added without removing existing lysine residues.

The C-terminus of the HBcAg has been shown to direct nuclear localization of this protein. (Eckhardt *et al.*, *J. Virol.* 65:575-582 (1991)). Further, this region of the protein is also believed to confer upon the HBcAg the ability to bind nucleic acids.

As indicated, HBcAgs suitable for use in the practice of the present invention also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus. However, variants of virus-like particles containing internal deletions within the sequence of the subunit composing the virus-like particle are also suitable in accordance with the present invention, provided their compatibility with the ordered or particulate structure of the virus-like particle. For example, internal deletions within the sequence of the HBcAg are suitable (Preikschat, P., *et al.*, *J. Gen. Virol.* 80:1777-1788 (1999)).

Further HBcAgs suitable for use in the practice of the present invention include N- and C-terminal truncation mutants. Suitable truncation

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mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, 35, 36, 37, 38, 39, 40, 41, 42 or 48 amino acids have been removed from the C-terminus.

Vaccine compositions of the invention can comprise mixtures of different HBcAgs. Thus, these vaccine compositions can be composed of HBcAgs which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (*e.g.*, deleted, inserted or substituted). In most applications, however, only one type of a HBcAg will be used.

The present invention is applicable to a wide variety of antigens. In a preferred embodiment, the antigen is a protein, polypeptide or peptide. In another embodiment the antigen is DNA. The antigen can also be a lipid, a carbohydrate, or an organic molecule, in particular a small organic molecule such as nicotine.

Antigens of the invention can be selected from the group consisting of the following: (a) polypeptides suited to induce an immune response against cancer cells; (b) polypeptides suited to induce an immune response against infectious diseases; (c) polypeptides suited to induce an immune response against allergens; (d) polypeptides suited to induce an immune response in farm animals or pets; and (e) fragments (*e.g.*, a domain) of any of the polypeptides set out in (a)-(d).

Preferred antigens include those from a pathogen (*e.g.* virus, bacterium, parasite, fungus) and tumors (especially tumor-associated antigens or "tumor markers"). Other preferred antigens are autoantigens.

In the specific embodiments described in the Examples, the antigen is the peptide p33 derived from lymphocytic choriomeningitis virus (LCMV). The p33 peptide represents one of the best studied CTL epitopes (Pircher *et al.*, "Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen," *Nature* 342:559 (1989); Tissot *et al.*, "Characterizing the

functionality of recombinant T-cell receptors in vitro: a pMHC tetramer based approach," *J Immunol Methods* 236:147 (2000); Bachmann *et al.*, "Four types of Ca<sup>2+</sup>-signals after stimulation of naive T cells with T cell agonists, partial agonists and antagonists," *Eur. J. Immunol.* 27:3414 (1997); Bachmann *et al.*, "Functional maturation of an anti-viral cytotoxic T cell response," *J. Virol.* 71:5764 (1997); Bachmann *et al.*, "Peptide induced TCR-down regulation on naive T cell predicts agonist/partial agonist properties and strictly correlates with T cell activation," *Eur. J. Immunol.* 27:2195 (1997); Bachmann *et al.*, "Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation," *Immunity* 7:549 (1997)). p33-specific T cells have been shown to induce lethal diabetic disease in transgenic mice (Ohashi *et al.*, "Ablation of 'tolerance' and induction of diabetes by virus infection in viral antigen transgenic mice," *Cell* 65:305 (1991)) as well as to be able to prevent growth of tumor cells expressing p33 (Kündig *et al.*, "Fibroblasts act as efficient antigen-presenting cells in lymphoid organs," *Science* 268:1343 (1995); Speiser *et al.*, "CTL tumor therapy specific for an endogenous antigen does not cause autoimmune disease," *J. Exp. Med.* 186:645 (1997)). This specific epitope, therefore, is particularly well suited to study autoimmunity, tumor immunology as well as viral diseases.

In one specific embodiment of the invention, the antigen or antigenic determinant is one that is useful for the prevention of infectious disease. Such treatment will be useful to treat a wide variety of infectious diseases affecting a wide range of hosts, *e.g.*, human, cow, sheep, pig, dog, cat, other mammalian species and non-mammalian species as well. Treatable infectious diseases are well known to those skilled in the art, and examples include infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, Papilloma virus etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc. Thus, antigens or antigenic determinants selected for the compositions of the invention will be well known to those in

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the medical art; examples of antigens or antigenic determinants include the following: the HIV antigens gp140 and gp160; the influenza antigens hemagglutinin, M2 protein and neuraminidase, Hepatitis B surface antigen or core and circumsporozoite protein of malaria or fragments thereof.

As discussed above, antigens include infectious microbes such as viruses, bacteria and fungi and fragments thereof, derived from natural sources or synthetically. Infectious viruses of both human and non-human vertebrates include retroviruses, RNA viruses and DNA viruses. The group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of RNA viruses that are antigens in vertebrate animals include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and

Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picomaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A, C, D, E and G viruses, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Aphovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus,

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Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest *virus*), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial *virus* and Pneumonia virus of mice); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses and filoviruses (Marburg virus and Ebola virus); the family Arenaviridae, including Lymphocytic

choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family Coronaviridae, including Infectious Bronchitis Virus (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine, monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A, B, C, D and E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotropic papilloma virus);



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the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc.). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Each of the foregoing lists is illustrative, and is not intended to be limiting.

In a specific embodiment of the invention, the antigen comprises one or more cytotoxic T cell epitopes, Th cell epitopes, or a combination of the two epitopes.

In addition to enhancing an antigen specific immune response in humans, the methods of the preferred embodiments are particularly well suited for treatment of other mammals or other animals, *e.g.*, birds such as hens, chickens, turkeys, ducks, geese, quail and pheasant. Birds are prime targets for many types of infections.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa *et al.*, *Avian Dis.* 23:366-385 (1979)). Since that time, CIAV has been detected in commercial poultry in all major poultry producing countries (van Bulow *et al.*, pp. 690-699 in "Diseases of Poultry", 9th edition, Iowa State University Press 1991).

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism and between 14-18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine can be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, in ovo or by other methods described herein.

Cattle and livestock are also susceptible to infection. Disease which affect these animals can produce severe economic losses, especially amongst

cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although Pestiviruses were previously classified in the Togaviridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups.

Equine herpesviruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms including visna-maedi.

Primates such as monkeys, apes and macaques can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott *et al.*, *Lancet* 36:1538-1541 (1990); Desrosiers *et al.*, *PNAS USA* 86:6353-6357 (1989); Murphey-Corb *et al.*, *Science* 246:1293-1297 (1989); and Carlson *et al.*, *AIDS Res. Human Retroviruses* 6:1239-1246 (1990)). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman *et al.*, *Nature* 345:622-625 (1990)).

Cats, both domestic and wild, are susceptible to infection with a variety of microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and

other types of pathogenic organisms in cats, the methods of the invention can be used to vaccinate cats to prevent them against infection.

Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia *virus* (FeLV), feline sarcoma *virus* (FeSV), endogenous type C oncomavirus (RD-114), and feline syncytia-forming *virus* (FeSFV). The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen *et al.*, *Science* 235:790-793 (1987). Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat and pallas cat.

Viral and bacterial diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity. Nucleic acid based vaccinations of fish are described, for example, in U.S. Patent No. 5,780,448.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered orally or by immersion or injection.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous fish, such as, for example, salmonids, carp, catfish, yellowtail, seabream and seabass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon and Arctic char. Examples of

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shellfish include, but are not limited to, clams, lobster, shrimp, crab and oysters. Other cultured aquatic animals include, but are not limited to, eels, squid and octopi.

Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein or nucleoprotein of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegumin or capsid protein or glycoprotein of channel catfish virus (CCV).

Polypeptides of bacterial pathogens include but are not limited to an iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of *Aeromonis salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytotoxin (mpr), a surface expressed hemolysin (ish), and a flagellar antigen of *Yersinia*; an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of Pasteurellosis; an OMP and a flagellar protein of *Vibrio anguillarum* and *V. ordalii*; a flagellar protein, an OMP protein, aroA, and purA of *Edwardsiella ictaluri* and *E. tarda*; and surface antigen of *Ichthyophthirius*; and a structural and regulatory protein of *Cytophaga columnari*; and a structural and regulatory protein of *Rickettsia*.

Polypeptides of a parasitic pathogen include but are not limited to the surface antigens of *Ichthyophthirius*.

In another aspect of the invention, there is provided vaccine compositions suitable for use in methods for preventing and/or attenuating diseases or conditions which are caused or exacerbated by "self" gene products (e.g., tumor necrosis factors). Thus, vaccine compositions of the invention include compositions which lead to the production of antibodies that prevent and/or attenuate diseases or conditions caused or exacerbated by "self" gene

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products. Examples of such diseases or conditions include graft versus host disease, IgE-mediated allergic reactions, anaphylaxis, adult respiratory distress syndrome, Crohn's disease, allergic asthma, acute lymphoblastic leukemia (ALL), non-Hodgkin's lymphoma (NHL), Graves' disease, systemic lupus erythematosus (SLE), inflammatory autoimmune diseases, myasthenia gravis, immunoproliferative disease lymphadenopathy (IPL), angioimmunoproliferative lymphadenopathy (AIL), immunoblastic lymphadenopathy (IBL), rheumatoid arthritis, diabetes, prion diseases, multiple sclerosis, Alzheimer disease and osteoporosis.

In related specific embodiments, compositions of the invention are an immunotherapeutic that can be used for the treatment and/or prevention of allergies, cancer or drug addiction.

The selection of antigens or antigenic determinants for the preparation of compositions and for use in methods of treatment for allergies would be known to those skilled in the medical arts treating such disorders. Representative examples of such antigens or antigenic determinants include the following: bee venom phospholipase A<sub>2</sub>, Bet v I (birch pollen allergen), 5 Dol m V (white-faced hornet venom allergen), and Der p I (House dust mite allergen), as well as fragments of each which can be used to elicit immunological responses.

The selection of antigens or antigenic determinants for compositions and methods of treatment for cancer would be known to those skilled in the medical arts treating such disorders (*see Renkvist et al., Cancer. Immunol. Immunother.* 50:3-15 (2001) which is incorporated by reference), and such antigens or antigenic determinants are included within the scope of the present invention. Representative examples of such types of antigens or antigenic determinants include the following: Her2 (breast cancer); GD2 (neuroblastoma); EGF-R (malignant glioblastoma); CEA (medullary thyroid cancer); CD52 (leukemia); human melanoma protein gp100; human melanoma protein gp100 epitopes such as amino acids 154-162 (sequence: KTWGQYWQV), 209-217 (ITDQVPFSV), 280-288 (YLEPGPVTA), 457-

466 (LLDGTATLRL) and 476–485 (VLYRYGSFSV); human melanoma protein melan-A/MART-1; human melanoma protein melan-A/MART-1 epitopes such as amino acids 27–35 (AAGIGILTV) and 32–40 (ILTVILGVL); tyrosinase and tyrosinase related proteins (*e.g.*, TRP-1 and TRP-2); tyrosinase epitopes such as amino acids 1–9 (MLLAVLYCL) and 369–377 (YMDGTMSQV); NA17-A nt protein; NA17-A nt protein epitopes such as amino acids 38–64 (VLPDVFIRC); MAGE-3 protein; MAGE-3 protein epitopes such as amino acids 271–279 (FLWGPRALV); other human tumors antigens, *e.g.* CEA epitopes such as amino acids 571–579 (YLSGANLNL); p53 protein; p53 protein epitopes such as amino acids 65–73 (RMPEAAPPV), 149–157 (STPPPGTRV) and 264–272 (LLGRNSFEV); Her2/neu epitopes such as amino acids 369–377 (KIFGSLAFL) and 654–662 (IISAVVGIL); NY-ESO-1 peptides 157-165 and 157-167, 159-167; HPV16 E7 protein; HPV16 E7 protein epitopes such as amino acids 86–93 (TLGIVCPI); as well as fragments of each which can be used to elicit immunological responses.

The selection of antigens or antigenic determinants for compositions and methods of treatment for drug addiction, in particular recreational drug addiction, would be known to those skilled in the medical arts treating such disorders. Representative examples of such antigens or antigenic determinants include, for example, opioids and morphine derivatives such as codeine, fentanyl, heroin, morphium and opium; stimulants such as amphetamine, cocaine, MDMA (methylenedioxymethamphetamine), methamphetamine, methylphenidate and nicotine; hallucinogens such as LSD, mescaline and psilocybin; as well as cannabinoids such as hashish and marijuana.

The selection of antigens or antigenic determinants for compositions and methods of treatment for other diseases or conditions associated with self antigens would be also known to those skilled in the medical arts treating such disorders. Representative examples of such antigens or antigenic determinants are, for example, lymphotoxins (*e.g.* Lymphotoxin  $\alpha$  (LT  $\alpha$ ), Lymphotoxin  $\beta$  (LT  $\beta$ )), and lymphotoxin receptors, Receptor activator of nuclear factor

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kappaB ligand (RANKL), vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGF-R), Interleukin 17 and amyloid beta peptide ( $A\beta_{1-42}$ ),  $TNF\alpha$ , MIF, MCP-1, SDF-1, Rank-L, M-CSF, Angiotensin II, Endoglin, Eotaxin, Grehlin, BLC, CCL21, IL-13, IL-17, IL-5, IL-8, IL-15, Bradykinin, Resistin, LHRH, GHRH, GIH, CRH, TRH and Gastrin, as well as fragments of each which can be used to elicit immunological responses.

In a particular embodiment of the invention, the antigen or antigenic determinant is selected from the group consisting of: (a) a recombinant polypeptide of HIV; (b) a recombinant polypeptide of Influenza virus (*e.g.*, an Influenza virus M2 polypeptide or a fragment thereof); (c) a recombinant polypeptide of Hepatitis C virus; (d) a recombinant polypeptide of Hepatitis B virus (e) a recombinant polypeptide of *Toxoplasma*; (f) a recombinant polypeptide of *Plasmodium falciparum*; (g) a recombinant polypeptide of *Plasmodium vivax*; (h) a recombinant polypeptide of *Plasmodium ovale*; (i) a recombinant polypeptide of *Plasmodium malariae*; (j) a recombinant polypeptide of breast cancer cells; (k) a recombinant polypeptide of kidney cancer cells; (l) a recombinant polypeptide of prostate cancer cells; (m) a recombinant polypeptide of skin cancer cells; (n) a recombinant polypeptide of brain cancer cells; (o) a recombinant polypeptide of leukemia cells; (p) a recombinant profiling; (q) a recombinant polypeptide of bee sting allergy; (r) a recombinant polypeptide of nut allergy; (s) a recombinant polypeptide of pollen; (t) a recombinant polypeptide of house-dust; (u) a recombinant polypeptide of cat or cat hair allergy; (v) a recombinant protein of food allergies; (w) a recombinant protein of asthma; (x) a recombinant protein of *Chlamydia*; and (y) a fragment of any of the proteins set out in (a)-(x).

In another embodiment of the present invention, the antigen, being coupled, fused or otherwise attached to the virus-like particle, is a T cell epitope, either a cytotoxic or a Th cell epitope. In a further preferred embodiment, the antigen is a combination of at least two, preferably different, epitopes, wherein the at least two epitopes are linked directly or by way of a

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linking sequence. These epitopes are preferably selected from the group consisting of cytotoxic and Th cell epitopes.

It should also be understood that a mosaic virus-like particle, *e.g.* a virus-like particle composed of subunits attached to different antigens and epitopes, respectively, is within the scope of the present invention. Such a composition of the present invention can be, for example, obtained by transforming *E.coli* with two compatible plasmids encoding the subunits composing the virus-like particle fused to different antigens and epitopes, respectively. In this instance, the mosaic virus-like particle is assembled either directly in the cell or after cell lysis. Moreover, such an inventive composition can also be obtained by attaching a mixture of different antigens and epitopes, respectively, to the isolated virus-like particle.

The antigen of the present invention, and in particular the indicated epitope or epitopes, can be synthesized or recombinantly expressed and coupled to the virus-like particle, or fused to the virus-like particle using recombinant DNA techniques. Exemplary procedures describing the attachment of antigens to virus-like particles are disclosed in WO 00/32227, in WO 01/85208 and in WO 02/056905, the disclosures of which are herewith incorporated by reference in its entirety.

The invention also provides a method of producing a composition for enhancing an immune response in an animal comprising a VLP and an immunostimulatory substance, preferably an unmethylated CpG-containing oligonucleotide bound to the VLP which comprises incubating the VLP with the immunostimulatory substance and oligonucleotide, respectively, adding RNase and purifying said composition. In an equally preferred embodiment, the method comprises incubating the VLP with RNase, adding the immunostimulatory substance and oligonucleotide, respectively, and purifying the composition. In one embodiment, the VLP is produced in a bacterial expression system. In another embodiment, the RNase is RNase A.

The invention further provides a method of producing a composition for enhancing an immune response in an animal comprising a VLP bound to



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an immunostimulatory substance, preferably to an unmethylated CpG-containing oligonucleotide which comprises disassembling the VLP, adding the immunostimulatory substance and oligonucleotide, respectively, and reassembling the VLP. The method can further comprise removing nucleic acids of the disassembled VLP and/or purifying the composition after reassembly.

The invention also provides vaccine compositions which can be used for preventing and/or attenuating diseases or conditions. Vaccine compositions of the invention comprise, or alternatively consist of, an immunologically effective amount of the inventive immune enhancing composition together with a pharmaceutically acceptable diluent, carrier or excipient. The vaccine can also optionally comprise an adjuvant.

The invention further provides vaccination methods for preventing and/or attenuating diseases or conditions in animals. In one embodiment, the invention provides vaccines for the prevention of infectious diseases in a wide range of animal species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines can be designed to treat infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc.

In another embodiment, the invention provides vaccines for the prevention of cancer in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines can be designed to treat all types of cancer including, but not limited to, lymphomas, carcinomas, sarcomas and melanomas.

As would be understood by one of ordinary skill in the art, when compositions of the invention are administered to an animal, they can be in a composition which contains salts, buffers, adjuvants or other substances which are desirable for improving the efficacy of the composition. Examples of

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materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including REMINGTON'S PHARMACEUTICAL SCIENCES (Osol, A, ed., Mack Publishing Co., (1990)).

Various adjuvants can be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Such adjuvants are also well known in the art. Further adjuvants that can be administered with the compositions of the invention include, but are not limited to, Monophosphoryl lipid, immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts, MF-59, and Virosomal adjuvant technology. The adjuvants can also comprise a mixture of these substances.

Compositions of the invention are said to be "pharmacologically acceptable" if their administration can be tolerated by a recipient individual. Further, the compositions of the invention will be administered in a "therapeutically effective amount" (*i.e.*, an amount that produces a desired physiological effect).

The compositions of the present invention can be administered by various methods known in the art. The particular mode selected will depend of course, upon the particular composition selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, can be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, parenteral, intracisternal, intravaginal, intraperitoneal, topical (as by powders, ointments, drops or transdermal patch), bucal, or as an oral or nasal spray. The term "parenteral" as used herein refers to modes of administration

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which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. The composition of the invention can also be injected directly in a lymph node.

Components of compositions for administration include sterile aqueous (*e.g.*, physiological saline) or non-aqueous solutions and suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption.

Combinations can be administered either concomitantly, *e.g.*, as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, *e.g.*, as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

Dosage levels depend on the mode of administration, the nature of the subject, and the quality of the carrier/adjuvant formulation. Typical amounts are in the range of about 0.1  $\mu\text{g}$  to about 20 mg per subject. Preferred amounts are at least about 1  $\mu\text{g}$  to about 100  $\mu\text{g}$  per subject. Multiple administration to immunize the subject is preferred, and protocols are those standard in the art adapted to the subject in question.

The compositions can conveniently be presented in unit dosage form and can be prepared by any of the methods well-known in the art of pharmacy. Methods include the step of bringing the compositions of the invention into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compositions of the invention into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

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Compositions suitable for oral administration can be presented as discrete units, such as capsules, tablets or lozenges, each containing a predetermined amount of the compositions of the invention. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, an elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compositions of the invention described above, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art.

Other embodiments of the invention include processes for the production of the compositions of the invention and methods of medical treatment for cancer and allergies using said compositions.

Further aspects and embodiments of the present invention will become apparent in the following examples and the appended claims.

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

All patents and publications referred to herein are expressly incorporated by reference in their entirety.

### *Example 1*

#### *Generation of p33-HBcAg VLPs.*

The DNA sequence of HBcAg containing peptide p33 from LCMV is given in Figure 1B. The p33-HBcAg VLPs (p33-VLPs) were generated as

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follows: Hepatitis B clone pEco63 containing the complete viral genome of Hepatitis B virus was purchased from ATCC. The gene encoding HBcAg was introduced into the EcoRI/HindIII restriction sites of expression vector pkk223.3 (Pharmacia) under the control of a strong tac promoter. The p33 peptide (KAVYNFATM) derived from lymphocytic choriomeningitis virus (LCMV) was fused to the C-terminus of HBcAg (1-185) via a three leucine-linker by standard PCR methods. A clone of *E. coli* K802 selected for good expression was transfected with the plasmid, and cells were grown and resuspended in 5 ml lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 30 mM NaCl, 10 mM EDTA, 0.25 % Tween-20, pH 7.0). 200 µl of lysozyme solution (20 mg/ml) was added. After sonication, 4 µl Benzonase and 10 mM MgCl<sub>2</sub> was added and the suspension was incubated for 30 minutes at RT, centrifuged for 15 minutes at 15,000 rpm at 4°C and the supernatant was retained.

Next, 20 % (w/v) (0.2 g/ml lysate) ammonium sulfate was added to the supernatant. After incubation for 30 minutes on ice and centrifugation for 15 minutes at 20,000 rpm at 4°C the supernatant was discarded and the pellet resuspended in 2-3 ml PBS. 20 ml of the PBS-solution was loaded onto a Sephacryl S-400 gel filtration column (Amersham Pharmacia Biotechnology AG), fractions were loaded onto a SDS-Page gel and fractions with purified p33-VLP capsids were pooled. Pooled fractions were loaded onto a Hydroxyapatite column. Flow through (which contains purified p33-VLP capsids) was collected (Figure 2B). Electron microscopy was performed according to standard protocols. A representative example is shown in Figure 2A.

### *Example 2*

#### *CpG-containing oligonucleotides can be packaged into HBcAg VLPs.*

Recombinant p33-VLPs were run on a native agarose (1%) gel electrophoresis and stained with ethidium bromide or Coomassie blue for the

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detection of RNA/DNA or protein (Figure 3). Bacterial produced VLPs contain high levels of single stranded RNA, which is presumably binding to the arginine repeats appearing near the C-terminus of the HBcAg protein and being geographically located inside the VLPs as shown by X-ray crystallography. The contaminating RNA can be easily digested and so eliminated by incubating the VLPs with RNase A. The highly active RNase A enzyme has a molecular weight of about 14 kDa and is presumably small enough to enter the VLPs to eliminate the undesired ribonucleic acids.

The recombinant p33-VLPs were supplemented with CpG-oligonucleotides (Figure 1A) before digestion with RNase A. As shown in Figure 4 the presence of CpG-oligonucleotides preserved the capsid structure as shown by similar migration compared to untreated p33-VLPs. The CpG-oligonucleotide-containing VLPs were purified from unbound oligonucleotides via dialysis (4500-fold dilution in PBS for 24 hours using a 300 kDa MWCO dialysis membrane) (Figure 5).

### *Example 3*

#### ***CpG-oligonucleotides can be packaged into VLPs by removal of the RNA with RNase and subsequent packaging of oligonucleotides into VLPs.***

The p33-VLPs (containing bacterial single-stranded RNA) were first incubated with RNase A to remove the RNA and in a second step the immunostimulating CpG-oligonucleotides (with normal phosphodiester bonds but also with phosphorothioate modification of the phosphate backbone) was supplemented to the samples (Figure 6). This experiment clearly shows that the CpG-oligonucleotides are not absolutely required simultaneously during the RNA degradation reaction but can be added at a later time.

#### *Example 4*

***VLPs containing CpG-oligonucleotides (with phosphorothioate modification of the phosphate backbone or normal phosphodiester bonds) induce enhanced anti-viral protection.***

Mice were subcutaneously primed with 100 µg CpG-oligonucleotide containing p33-VLPs. Before immunization, p33-VLP preparations were extensively purified from unbound CpG-oligonucleotides via dialysis (see Example 2 and Figure 5). As controls mice were subcutaneously primed with 100 µg p33-VLP alone, mixed with 20 nmol CpG-oligonucleotide, with 20 nmol CpG-oligonucleotide alone or left untreated. Twenty-one days later, mice were challenged with LCMV (200 pfu, intravenously) and viral titers were assessed in the spleens 5 days later as described in Bachmann, M. F., "Evaluation of lymphocytic choriomeningitis virus-specific cytotoxic T cell responses," in Immunology Methods Manual, Lefkowitz, I., ed., Academic Press Ltd., New York, NY (1997) p. 1921. The results are shown in Figures 7, 8 and 9.

#### *Example 5*

##### ***Generation of BKV polyoma capsids.***

BK virus (BKV) is a non-enveloped double stranded DNA virus belonging to the polyoma virus subfamily of the papovaviridae. VP1 is the major capsid protein. VP1 has 362 amino acids (Figure 10) and is 42 kDa in size. When produced in *E. coli*, insect cells or yeast VP1 spontaneously forms capsid structures (Salunke D.M., *et al.*, *Cell* 46(6):895-904 (1986); Sasnauskas, K., *et al.*, *Biol. Chem.* 380(3):381-6 (1999); Sasnauskas, K., *et al.*, 3<sup>rd</sup> International Workshop "Virus-like particles as vaccines" Berlin, September 26-29 (2001); Touze, A., *et al.*, *J Gen Virol.* 82(Pt 12):3005-9 (2001). The capsid is organized in 72 VP1 pentamers forming an icosahedral structure. The capsids have a diameter of approximately 45 nm.

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*Example 6****Fluorescein labeled CpG-containing oligonucleotides can be packaged into BKV VLPs.***

VLPs produced in yeast contain small amounts of RNA which can be easily digested and so eliminated by incubating the VLPs with RNase A. The highly active RNase A enzyme has a molecular weight of about 14 kDa and is small enough to enter the VLPs to eliminate the undesired ribonucleic acids. Recombinantly produced BKV VLPs were concentrated to 1 mg/ml in PBS buffer pH7.2 and incubated in the absence or presence of RNase A (200µg/ml, Roche Diagnostics Ltd, Switzerland) for 3 h at 37°C. After RNase A digestion BKV VLPs were supplemented with 75 nmol/ml fluorescein labeled phosphorothioate CpG-FAM oligonucleotide and incubated for 3 h at 37°C. Subsequently BKV VLPs were subjected to DNaseI digestion for 3 h at 37°C (40 u/ml AMPD1, Sigma, Division of Fluka AG, Switzerland) or loaded without DNaseI digestion. The samples were complemented with 6-fold concentrated DNA-loading buffer (10 mM Tris pH7.5, 10% v/v glycerol, 0.4% orange G) and run for 1 h at 65 volts in a 0.8% native tris-acetate pH 7.5 agarose gel.

Figure 12 shows BKV VLPs in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with fluorescent CpG-FAM oligonucleotides (oligonucleotide from Figure 1A with a 5'-fluorescein-label) upon staining with ethidium bromide or without ethidium bromide staining. In the presence of ethidium bromide nucleic acids are detected, while in its absence UV excitation leads to fluorescence of the fluorescein-label in the CpG-FAM.

The RNase A digestion leads to a change in migration of the VLP, visible on Coomassie stained agarose gel, presumably due to the lack of negative charges from the RNA (Figures 13 and 14). Addition of CpG-oligonucleotide restores the migration of BKV VLPs and results in a fluorescent band with the same migration as the RNA band present in



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untreated VLPs. This clearly shows that CpG-FAM oligonucleotides have been packaged into VLPs.

### *Example 7*

#### *Large double stranded oligonucleotides can be packaged into BKV VLPs.*

To introduce double stranded (ds) nucleotide sequences, the RNase A treated recombinant BKV VLPs (Example 6) were supplemented with 50µg/ml (ds) DNA fragments (246 bp in length, Figure 11) and incubated for 3 h at 37°C. The samples were complemented with 6-fold concentrated DNA-loading buffer (10 mM Tris pH8.0, 10% v/v glycerol, 0.4% orange G) and run for 1 h at 65 volts in a 0.8% native tris-acetate pH8.0 agarose gel.

Figure 13 shows BKV VLPs (15 µg) in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with (ds) DNA upon staining with ethidium bromide or Coomassie Blue in order to assess the presence of RNA/DNA or protein. Packaged DNA molecules are visible in the presence of ethidium bromide as a band with the same migration as the VLP band visualized with Coomassie Blue.

Addition of (ds) DNA restores the migration of BKV VLPs and results in a DNA band with the same migration as the Coomassie Blue stained VLPs. This clearly shows that (ds) DNA has been packaged into BKV VLPs.

### *Example 8*

#### *CpG-containing oligonucleotides can be packaged into BKV VLPs.*

To introduce immunostimulatory CpG-oligonucleotides, the RNase A treated recombinant BKV VLPs (Example 6) were supplemented with 150 nmol/ml CpG-oligonucleotides with phosphodiester backbone or with phosphorothioate backbone and incubated for 3 h at 37°C. VLP preparations

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for mouse immunization were extensively dialysed (10,000-fold diluted) for 24 h against PBS pH7.2 with a 300 kDa MWCO dialysis membrane (Spectrum Medical industries Inc., Houston, USA) to eliminate RNase A and the excess of CpG-oligonucleotides. The samples were complemented with 6-fold concentrated DNA-loading buffer (10 mM Tris pH7.5, 10% v/v glycerol, 0.4% orange G) and run for 1 h at 65 volts in a 0.8% native tris-acetate pH7.5 agarose gel.

Figure 14 shows BKV VLPs (15 µg) in a native 0.8% agarose gel electrophoresis after control incubation or after digestion with RNase A and subsequent incubation with CpG-oligonucleotides (with phosphodiester- or with phosphorothioate backbone) upon staining with ethidium bromide (A) or Coomassie Blue (B) in order to assess the presence of RNA/DNA or protein and the reduction of unbound CpG-oligonucleotides after dialysis. Unbound CpG-oligonucleotides are visible as a low molecular weight ethidium bromide stained band.

Addition of CpG-oligonucleotides restores the migration of BKV VLPs and results in a DNA band with the same migration as the Coomassie Blue stained VLPs. This clearly shows that CpG-oligonucleotides are packaged into BKV VLPs.

### ***Example 9***

***VLPs containing CpG-oligonucleotides (with phosphorothioate modification of the phosphate backbone) induce enhanced Th1 directed immune response.***

Female BALB/c mice (three mice per group) were subcutaneously injected with 10 µg BKV VLPs containing phosphorothioate CpG-oligonucleotide (Figure 1A). As controls mice were subcutaneously injected with either 10 µg of RNase treated BKV VLPs alone or BKV VLPs mixed with 0.3 nmol or 20 nmol phosphorothioate CpG-oligonucleotides in 200 µl PBS pH7.2 or were left untreated. BKV VLPs were prepared as described in

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Example 8 and before immunization extensively purified from unbound CpG-oligonucleotide by dialysis. On day 14 after immunization blood was taken and IgG1 and IgG2a antibody response to BKV VLPs was determined.

Figure 15 shows IgG1 and IgG2a antibody response to BKV VLPs on day 14 after immunization. Immunization with RNase A treated BKV VLPs containing phosphorothioate CpG-oligonucleotides results in a decreased IgG1 and an increased anti-BKV VLP IgG2a titer as compared to immunization with the same amount (0.3 nmol) of CpG-oligonucleotides mixed with BKV VLPs or BKV VLPs alone. Mice immunized with BKV VLPs mixed with 20 nmol phosphorothioate CpG-oligonucleotides show very low IgG1 and high IgG2a titers. The decrease in IgG1 titer and the increase in IgG2a titer as compared to controls demonstrates a Th1 cell directed immune response induced by phosphorothioate CpG-oligonucleotides packaged in BKV VLPs. Figure 15 clearly demonstrates the higher potency of BKV VLPs containing CpG-oligonucleotides packaged within the particles as compared to BKV VLPs simply mixed with CpG-oligonucleotides.

### *Example 10*

*Linear double-stranded DNA (dsDNA) can be packaged into VLPs by first RNase digestion and subsequently addition of dsDNA.*

The p33-VLPs preparations (containing bacterial RNA) (EXAMPLE 1) were first incubated with RNaseA to remove the RNA and in a second step the linear dsDNA (350 bp long) was supplemented to the samples (Figure 16). The migration of the p33-VLPs packaged with the dsDNA was similar to the one of p33-VLP containing RNA. This experiment shows that linear dsDNA of at least 350 base pairs in length can be packaged into the virus-like particles.

### *Example 11*

#### ***Immunostimulatory nucleic acids can be packaged into HBcAg VLPs comprising fusion proteins with antigens.***

HBcAg VLPs, when produced in *E. coli* by expressing the Hepatitis B core antigen fusion protein HBc33 (Example 1) or the fusion protein to the peptide P1A (HBcP1A), contain RNA which can be digested and so eliminated by incubating the VLPs with RNase A.

The gene *P1A* codes for a protein that is expressed by the mastocytoma tumor cell line P815. The dominant CTL epitope, termed P1A peptide, binds to MHC class I (Ld) and the complex is recognized by specific CTL clones (Brändle et al., 1998, Eur. J. Immunol. 28: 4010-4019). Fusion of peptide P1A-1 (LPYLGWLVF) to the C-terminus of HBcAg (aa 185, see Example 1) was performed by PCR using appropriate primers using standard molecular biology techniques. A three leucine linker was cloned between the HBcAg and the peptide sequence. Expression was performed as described in Example 1. The fusion protein of HBcAg with P1A, termed HBcP1A, formed capsids when expressed in *E. coli* which could be purified similar to the procedure described in Example 1.

***Enzymatic RNA hydrolysis:*** Recombinantly produced HBcAg-p33 (HBc33) and HBcAg-P1A (HBcP1A) VLPs at a concentration of 1.0 mg/ml in 1 x PBS buffer (KCl 0.2g/L, KH<sub>2</sub>PO<sub>4</sub> 0.2g/L, NaCl 8 g/L, Na<sub>2</sub>HPO<sub>4</sub> 1.15 g/L) pH 7.4, were incubated in the presence of 300 µg/ml RNase A (Qiagen AG, Switzerland) for 3 h at 37°C in a thermomixer at 650 rpm.

***Packaging of immunostimulatory nucleic acids:*** After RNA digestion with RNase A HBcAg-p33 VLPs were supplemented with 130 nmol/ml CpG-oligonucleotides B-CpG, NKCPG, G10-PO (Table I). Similarly, the 150mer single-stranded Cy150-1 and 253mer double stranded dsCyCpG-253, both containing multiple copies of CpG motifs, were added at 130 nmol/ml or 1.2 nmol/ml, respectively, and incubated in a thermomixer for 3 h at 37°C. Double stranded CyCpG-253 DNA was produced by cloning a double stranded

multimer of CyCpG into the EcoRV site of pBluescript KS-. The resulting plasmid, produced in *E. coli* XL1-blue and isolated using the Qiagen Endofree plasmid Giga Kit, was digested with restriction endonucleases XhoI and XbaI and resulting restriction products were separated by agarose electrophoresis. The 253 bp insert was isolated by electro-elution and ethanol precipitation. Sequence was verified by sequencing of both strands.

Table I: Sequences of immunostimulatory nucleic acids used in the Examples.

Small letters indicate deoxynucleotides connected via phosphorothioate bonds while larger letters indicate deoxynucleotides connected via phosphodiester bonds

CyCpGpt	tccatgacgttcctgaataat
CyCpG	TCCATGACGTTCCTGAATAAT
B-CpGpt	tccatgacgttcctgacgtt
B-CpG	TCCATGACGTTCCTGACGTT
NKCpGpt	ggGGTCAACGTTGAggggg
NKCpG	GGGGTCAACGTTGAGGGGG
CyCpG-rev-pt	attattcaggaacgtcatgga
g10gacga-PO (G10-PO)	GGGGGGGGGGGACGATCGTCGGGGGGGGGG
g10gacga-PS (G10-PS)	gggggggggggacgatcgtcggggggggggg
(CpG) 20OpA	CGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG CGCGAAATGCA TGTCAAAGACAGCAT
Cy (CpG) 20	TCCATGACGTTCCTGAATAATCGCGCGCGCGCGCG GCGCGCGCGCG CGCGCGCGCGCGCG
Cy (CpG) 20-OpA	TCCATGACGTTCCTGAATAATCGCGCGCGCGCGCG GCGCGCGCGCG CGCGCGCGCGCGCGAAATGCATGTCAAAGACAGCA
CyOpA	TCCATGACGTTCCTGAATAATAATGCATGTCAA ACAGCAT
CyCyCy	TCCATGACGTTCCTGAATAATTCCATGACGTTCC AATAATTCCAT GACGTTTCCTGAATAAT
Cy150-1	TCCATGACGTTCCTGAATAATTCCATGACGTTCC AATAATTCCAT GACGTTTCCTGAATAATTGGATGACGTTGGTGAATA TTCCATGACGT TCCTGAATAATTCCATGACGTTTCCTGAATAATTCC TGACGTTTCCTG

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	AATAATTCC
dsCyCpG-253 (complementary strand r shown)	CTAGAACTAGTGGATCCCCGGGCTGCAGGAATT ATTCATGACTT CCTGAATAATTCCATGACGTTGGTGAATAATTCC GACGTTCTGA ATAATTCCATGACGTTCTGAATAATTCCATGAC TCCTGAATAAT TCCATGACGTTCTGAATAATTCCATGACGTTCC AATAATTCCAT GACGTTCTGAATAATTCCATGACGTTCTGAAA TCCAATCAAGC TTATCGATACCGTCGACC

*DNase I treatment:* Packaged HBcAg-p33 VLPs were subsequently subjected to DNaseI digestion (5 U/ml) for 3 h at 37°C (DNaseI, RNase free Fluka AG, Switzerland) and were extensively dialysed (2 x against 200-fold volume) for 24 h against PBS pH 7.4 with a 300 kDa MWCO dialysis membrane (Spectrum Medical industries Inc., Houston, USA) to eliminate RNase A and the excess of CpG-oligonucleotides.

*Benzonase treatment:* Since some single stranded oligodeoxynucleotides were partially resistant to DNaseI treatment, Benzonase treatment was used to eliminate free oligonucleotides from the preparation. 100-120 U/ml Benzonase (Merck KGaA, Darmstadt, Germany) and 5 mM MgCl<sub>2</sub> were added and incubated for 3 h at 37°C before dialysis.

*Dialysis:* VLP preparations packaged with immunostimulatory nucleic acids used in mouse immunization experiments were extensively dialysed (2x against 200fold volume) for 24 h against PBS pH 7.4 with a 300 kDa MWCO dialysis membrane (Spectrum Medical Industries, Houston, US) to eliminate added enzymes and free nucleic acids.

*Analytics of packaging: release of packaged immunostimulatory nucleic acids:* To 50 µl capsid solution 1 µl of proteinase K (600 U/ml, Roche, Mannheim, Germany), 3 µl 10% SDS-solution and 6 µl 10fold proteinase buffer (0.5 M NaCl, 50 mM EDTA, 0.1 M Tris pH 7.4) were added and subsequently incubated overnight at 37°C. VLPs are completely hydrolysed under these conditions. Proteinase K was inactivated by heating for 20 min at

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65°C. 1 µl RNase A (Qiagen, 100 µg/ml, diluted 250 fold) was added to 25 µl of capsid. 2-30 µg of capsid were mixed with 1 volume of 2x loading buffer (1xTBE, 42% w/v urea, 12% w/v Ficoll, 0.01 % Bromphenolblue), heated for 3 min at 95°C and loaded on a 10% (for oligonucleotides of about 20 nt length) or 15% (for > than 40 mer nucleic acids) TBE/urea polyacrylamid gel (Invitrogen). Alternatively samples were loaded on a 1% agarose gel with 6x loading dye (10 mM Tris pH 7.5, 50 mM EDTA, 10% v/v glycerol, 0.4 % orange G). TBE/urea gels were stained with CYBRGold and agarose gels with stained with ethidium bromide.

Figures 17, 18 and 19 show the packaging of B-CpG, NKCPG and G10-PO oligonucleotides into HBc33. RNA content in the VLPs was strongly reduced after RNaseA treatment (Figure 17A, 18A, 19A) while most of the capsid migrated as a a slow migrating smear presumably due to the removal of the negatively charged RNA (Figure 17B, 18B, 19B). After incubation with an excess of oligonucleotid the capsids contained a higher amount of nucleic acid than the RNaseA treated capsids and therefore migrated at similar velocity as the untreated capsids. Additional treatment with DNase I or Benzonase degraded the free oligonucleotides while oligonucleotides packaged in the capsids did not degrade, clearly showing packaging of oligonucleotides. In some cases packaging of oligonucleotides was confirmed by proteinase K digestion (as described in Examples 15 and 16) after DNaseI/Benzonase treatment and dialysis. The finding that oligonucleotides released from the capsid with the procedure described above were of the same size than the oligonucleotide used for packaging clearly demonstrated packaging of oligonucleotides (Figure 17C, 18C).

Figure 20 shows packaging of a large single-stranded oligonucleotide Cy150-1 into HBc33. Cy150-1 contains 7.5 repeats of CyCpG and was synthesized according standard oligonucleotide synthesis methods (IBA, Göttingen, Germany). RNA content in the capsid was strongly reduced after RNaseA treatment while most of the capsid migrated as a slow migrating smear (Figure 20A, B). Capsid were diluted with 4 volumes of water and

concentrated to 1 mg/ml. After incubation with an excess of Cy150-1 the capsid contained a bigger amount of nucleic acid and thus migrated at similar velocity as the untreated capsids. Additional treatment with DNaseI degraded the free, not packaged oligonucleotides while oligonucleotides in capsids were not degraded (Figure 20A). Release of the DNaseI-resistant nucleic acid from the packaged VLPs by heating for 3 min at 95°C in TBE/urea loading buffer revealed the presence of the 150 mer (Figure 20 C).

Figure 21 shows packaging of oligonucleotide NKCPgt in HBcP1A. Treatment with RNase reduced nucleic acid content and slowed migration of the capsids. Addition of NKCPgt restored nucleic acid content in capsids and fast migration.

Figure 17 depicts the analysis of B-CpG packaging into HBc33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 50 µg of the following samples: 1. HBc33 VLP untreated; 2. HBc33 VLP treated with RNase A; 3. HBc33 VLP treated with RNase A and packaged with B-CpG; 4. HBc33 VLP treated with RNase A, packaged with B-CpG and treated with DNaseI; 5. HBc33 VLP treated with RNase A, packaged with B-CpG, treated with DNaseI and dialysed; 6. 1 kb MBI Fermentas DNA ladder. (C) depicts the analysis of the amount of packaged oligo extracted from the VLP on a 1.5% agarose gel stained with ethidium bromide: Loaded on gel are the following samples: 1. 0.5 nmol B-CpG control; 2. 0.5 nmol B-CpG control; 3. B-CpG oligo content HBc33 after phenol / chloroform extraction; 4. B-CpG oligo content HBc33 after phenol / chloroform extraction and RNase A treatment; 5. B-CpG oligo content HBc33 after phenol/ chloroform extraction and DNaseI treatment; 6. empty; 7. MBI Fermentas 100 bp DNA ladder

Figure 18 depicts the analysis of NKCPG packaging into HBc33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 15 µg of the following samples: 1. HBc33 VLP untreated; 2. HBc33 VLP treated with RNase A; 3. HBc33 VLP treated with RNase A and packaged with NKCPG; 4. HBc33 VLP treated with RNase A,



packaged with NKCPG, treated with DNaseI and dialysed; 5. 1 kb MBI Fermentas DNA ladder. (C) depicts the analysis of the amount of packaged oligo extracted from the VLP on a 15% TBE/urea gel stained with CYBR Gold. Loaded on gel are the following samples: 1. NKCPG oligo content HBc33 after proteinase K digestion and RNase A treatment; 2. 20 pmol NKCPG control; 3. 10 pmol NKCPG control; 4. 40 pmol NKCPG control

Figure 19 depicts the analysis of g10gacga-PO packaging into HBc33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 15 µg of the following samples: 1. 1 kb MBI Fermentas DNA ladder; 2. HBc33 VLP untreated; 3. HBc33 VLP treated with RNase A; 4. HBc33 VLP treated with RNase A and packaged with g10gacga-PO; 5. HBc33 VLP treated with RNase A, packaged with g10gacga-PO, treated with Benzonase and dialysed.

Figure 20 depicts the analysis of CyCpG-150 packaging into HBc33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 15 µg of the following samples: 1. 1 kb MBI Fermentas DNA ladder; 2. HBc33 VLP untreated; 3. HBc33 VLP treated with RNase A; 4. HBc33 VLP treated with RNase A and packaged with CyCpG-150; 5. HBc33 VLP treated with RNase A, packaged with CyCpG-150, treated with DNaseI and dialysed; 6. HBc33 VLP treated with RNase A, packaged with CyCpG-150, treated with DNaseI and dialysed. (C) depicts the analysis of the amount of packaged oligo extracted from the VLP on a 10 % TBE/urea gel stained with CYBR Gold. Loaded on gel are the following samples: 1. 20 pmol CyCpG-150 control; 2. 10 pmol CyCpG-150 control; 3. 4 pmol CyCpG-150 control; 4. CyCpG-150 oligo content of 4 µg HBc33 after 3 min at 95°C with 1 volume TBE/urea sample buffer.

Figure 21 depicts the analysis of NKCPGpt packaging into HBcP1A VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 15 µg of the following samples: 1. 1 kb MBI Fermentas DNA ladder; 2. HBcP1A VLP untreated; 3. HBcP1A VLP treated

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with RNase A; 4. HBcP1A VLP treated with RNase A and packaged with NKCpGpt.

### *Example 12*

#### ***Immunostimulatory nucleic acids can be packaged in HBcAg-wt coupled with antigens.***

Recombinantly produced HBcAg-wt VLPs were packaged after coupling with peptide p33 (CGG-KAVYNFATM), derived from lymphocytic choriomeningitis virus (LCMV). For coupling HBcAg-wt VLPs (2 mg/ml) were derivatized with 25x molar excess of SMPH (Succinimidyl-6-[( $\beta$ -maleimido-propionamido)hexanoate], Pierce) for 1 h at 25°C in a thermomixer. The derivatized VLPs were dialyzed to Mes buffer (2-(N-morpholino) ethanesulphonic acid) pH 7.4 for 2 x 2 h using MWCO 10.000 kD dialysis membranes at 4°C. VLPs (50 $\mu$ M) were subsequently coupled to the N-terminal cysteine of the p33 peptide (250 $\mu$ M) during a 2 h incubation in a thermomixer at 25°C. Samples were dialyzed (MWCO 300.000) extensively to 1x PBS pH 7.4 to eliminate undesired free peptide.

Figure 22 shows SDS-PAGE analysis of HBcAg wt VLPs derivatization with SMPH and coupling to p33 peptide. Samples were analysed by 16% SDS PAGE and stained with Coomassie Blue. HBcAg-wt was visible as a 21 kD protein band. Due to the low molecular weight of SMPH is the derivatised product only slightly larger and can not be distinguished by SDS-PAGE. Peptide alone was visible as a 3 kD band and coupled product, termed HBx33, showed a strong secondary band at approximately 24 kD accounting for more than 50% of total HBcAg-wt.

*Enzymatic RNA hydrolysis:* HBx33 VLPs (0.5-1.0 mg/ml, 1xPBS buffer pH7.4) in the presence of RNase A (300  $\mu$ g/ml, Qiagen AG, Switzerland) were diluted with 4 volumes H<sub>2</sub>O to decrease salt concentration to a final 0.2xPBS concentration and incubated for 3 h at 37°C in a thermomixer at 650 rpm.

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*Packaging of immunostimulatory nucleic acids:* After RNase A digestion HBx33 VLPs were concentrated using Millipore Microcon or Centriplus concentrators, then supplemented with 130 nmol/ml CpG-oligonucleotide B-CpGpt and incubated in a thermomixer for 3 h at 37°C in 0.2xPBS pH 7.4. Subsequently, reaction mixtures were subjected to DNaseI digestion (5 U/ml) for 3 h at 37°C (DNaseI, RNase free Fluka AG, Switzerland). VLP preparations for mouse immunization were extensively dialysed (2x against 200-fold volume) for 24 h against PBS pH 7.4 with a 300 kDa MWCO dialysis membrane (Spectrum Medical industries Inc., Houston, USA) to eliminate RNase A and the excess of CpG-oligonucleotides. Figure 23 shows that RNase treatment reduced the nucleic acid content of the capsids and slowed their migration. Addition of B-CpGpt restored nucleic acid content and fast migration of capsids. DNase I only digested the free oligonucleotides while the packaged oligonucleotides remained in the VLP also after dialysis (Figure 23).

Figure 22 depicts the SDS-PAGE analysis of the p33 coupling to HBc VLPs after Coomassie Blue staining. Loaded on the gel are the following samples: 1. NEB Prestained Protein Marker, Broad Range (# 7708S), 10 µl; 2. CGG-p33 peptide; 3. HBc VLP derivatized with SMPH, before dialysis; 4. HBc VLP derivatized with SMPH, after dialysis; 5. HBc VLP coupled with CGG-p33, supernatant; 6. HBc VLP coupled with CGG-p33, pellet.

Figure 23 depicts the analysis of B-CpGpt packaging into HBx33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 50 µg of the following samples: 1. HBx33 VLP untreated; 2. HBx33 VLP treated with RNase A; 3. HBx33 VLP treated with RNase A and packaged with B-CpGpt; 4. HBx33 VLP treated with RNase A, packaged with B-CpGpt and treated with DNaseI; 5. HBx33 VLP treated with RNase A, packaged with B-CpGpt, treated with DNaseI and dialysed; 6. 1 kb MBI Fermentas DNA ladder

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### ***Example 13***

#### ***Immunostimulatory nucleic acids can be packaged into Q $\beta$ VLPs coupled with antigens.***

##### ***Coupling of p33 peptides to Q $\beta$ VLPs:***

Recombinantly produced Q $\beta$  VLPs were used after coupling to p33 peptides containing an N-terminal CGG or and C-terminal GGC extension (CGG-KAVYNFATM and KAVYNFATM-GGC). Recombinantly produced Q $\beta$  VLPs were derivatized with a 10 molar excess of SMPH (Pierce) for 0.5 h at 25°C, followed by dialysis against 20 mM HEPES, 150 mM NaCl, pH 7.2 at 4°C to remove unreacted SMPH. Peptides were added in a 5 fold molar excess and allowed to react for 2 h in a thermomixer at 25°C in the presence of 30% acetonitrile. Figure 24 shows the SDS-PAGE analysis demonstrating multiple coupling bands consisting of one, two or three peptides coupled to the Q $\beta$  monomer (Arrows, Figure 24).

Q $\beta$  VLPs, when produced in *E. coli* by expressing the bacteriophage Q $\beta$  capsid protein, contain RNA which can be digested and so eliminated by incubating the VLPs with RNase A.

##### ***Low ionic strength and low Q $\beta$ concentration allow RNA hydrolysis of Q $\beta$ VLPs by RNase A:***

Q $\beta$  VLPs at a concentration of 1.0 mg/ml in 20mM Hepes/150mM NaCl buffer (HBS) pH 7.4 were either digested directly by addition of RNase A (300  $\mu$ g/ml, Qiagen AG, Switzerland) or were diluted with 4 volumes H<sub>2</sub>O to a final 0.2 x HBS concentration and then incubated with RNase A (60  $\mu$ g/ml, Qiagen AG, Switzerland). Incubation was allowed for 3 h at 37°C in a thermomixer at 650 rpm. Figure 25 demonstrates that in 1xHBS only a very weak reduction of RNA content was observed, while in 0.2x HBS most of the RNA were hydrolysed. In agreement, capsid migration was unchanged after

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addition of RNase A in 1x HBS, while migration was slower after addition of RNase in 0.2xHBS (Figure 25 B,D).

*Low ionic strength increases nucleic acid packaging in Q $\beta$  VLPs:*

After RNase A digestion in 0.2 x HBS the Q $\beta$  VLPs were concentrated to 1 mg/ml using Millipore Microcon or Centriplus concentrators and aliquots were dialysed against 1x HBS or 0.2 x HBS. Q $\beta$  VLPs were supplemented with 130 nmol/ml CpG-oligonucleotide B-CpG and incubated in a thermomixer for 3 h at 37°C. Subsequently Q $\beta$  VLPs were subjected to Benzonase digestion (100 U/ml) for 3 h at 37°C. Samples were analysed on 1% agarose gels after staining with ethidium bromide or Coomassie Blue. Figure 26 shows that in 1x HBS only a very low amount of oligonucleotides could be packaged, while in 0.2 x HBS a strong ethidium bromide stained band was detectable, which colocalized with the Coomassie blue stain of the capsids.

*Different immunostimulatory nucleic acids can be packaged in Q $\beta$  VLPs:*

After RNase A digestion in 0.2 x HBS the Q $\beta$  VLPs were concentrated to 1 mg/ml using Millipore Microcon or Centriplus concentrators and supplemented with 130 nmol/ml CpG-oligonucleotides B-CpGpt, g10gacga and the 253 mer dsCyCpG-253 (Table I) and incubated in a thermomixer for 3 h at 37°C. Subsequently Q $\beta$  VLPs were subjected to DNase I digestion (5 U/ml) or Benzonase digestion (100 U/ml) for 3 h at 37°C. Samples were analysed on 1% agarose gels after staining with ethidium bromide or Coomassie Blue. Figure 27 shows that the different nucleic acids B-CpGpt, g10gacga and the 253mer dsDNA could be packaged into Q $\beta$ 33. Packaged nucleic acids were resistant to DNase I digestion and remained packaged during dialysis (Figure 27). Packaging of B-CpGpt was confirmed by release

of the nucleic acid by proteinase K digestion followed by agarose electrophoresis and ethidium bromide staining (Figure 27C).

Figure 24 depicts the SDS-PAGE analysis of the p33 coupling to Q $\beta$  VLPs after Coomassie Blue staining. Loaded are the following samples: (A) 1. NEB Prestained Protein Marker, Broad Range (# 7708S), 10  $\mu$ l; 2. Q $\beta$  VLP, 14  $\mu$ g; 3. Q $\beta$  VLP derivatized with SMPH, after dialysis; 4. Q $\beta$  VLP coupled with CGG-p33, supernatant. (B) 1. NEB Prestained Protein Marker, Broad Range (# 7708S), 10  $\mu$ l; 2. Q $\beta$  VLP, 10  $\mu$ g; 3. Q $\beta$  VLP coupled with GGC-p33, supernatant.

Figure 25 depicts the analysis of RNA hydrolysis from Q $\beta$  VLPs by RNase A under low and high ionic strength on a 1% agarose gel stained with ethidium bromide (A, C) and Coomassie Blue (B, D). Loaded on the gel are the following samples: (A, B) 1. MBI Fermentas 1kb DNA ladder; 2. Q $\beta$  VLP untreated; 3. Q $\beta$  VLP treated with RNase A in 1x HBS buffer pH7.2. (C, D) 1. MBI Fermentas 1kb DNA ladder; 2. Q $\beta$  VLP untreated; 3. Q $\beta$  VLP treated with RNase A in 0.2 x HBS buffer pH7.2.

Figure 26 depicts the analysis of B-CpG packaging into Q $\beta$  VLPs under low and high ionic strength on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are the following samples: 1. Q $\beta$  VLP untreated; 2. Q $\beta$  VLP treated with RNase A; 3. Q $\beta$  VLP treated with RNase A and packaged with B-CpG in 0.2x HBS buffer pH7.2 and treated with Benzonase; 4. HBx33 VLP treated with RNase A, packaged with B-CpG in 1x HBS buffer pH7.2 and treated with Benzonase.

Figure 27 depicts the analysis of B-CpGpt packaging into Qbx33 VLPs on a 1% agarose gel stained with ethidium bromide (A) and Coomassie Blue (B). Loaded on the gel are 50  $\mu$ g of the following samples: 1. Qbx33 VLP untreated; 2. Qbx33 VLP treated with RNase A; 3. Qbx33 VLP treated with RNase A and packaged with B-CpGpt; 4. Qbx33 VLP treated with RNase A, packaged with B-CpGpt, treated with DNaseI and dialysed; 5. 1 kb MBI Fermentas DNA ladder. (C) depicts the analysis of the amount of packaged

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oligo extracted from the VLP on a 15% TBE/urea stained with CYBR Gold. Loaded on gel are the following samples: 1. BCpGpt oligo content of 2 µg Qbx33 VLP after proteinase K digestion and RNase A treatment; 2. 20 pmol BCpGpt control; 3. 10 pmol BCpGpt control; 4. 5 pmol BCpGpt control

Figure 27 D and E depict the analysis of g10gacga-PO packaging into Qbx33 VLPs on a 1% agarose gel stained with ethidium bromide (D) and Coomassie Blue (E). Loaded on the gel are 15 µg of the following samples: 1. MBI Fermentas 1 kb DNA ladder; 2. Qbx33 VLP untreated; 3. Qbx33 VLP treated with RNase A; 4. Qbx33 VLP treated with RNase A and packaged with g10gacga-PO; 5. Qbx33 VLP treated with RNase A, packaged with g10gacga-PO, treated with Benzonase and dialysed.

Figure 27 E and F depict the analysis of dsCyCpG-253 packaging into Qbx33 VLPs on a 1% agarose gel stained with ethidium bromide (E) and Coomassie Blue (F). Loaded on the gel are 15 µg of the following samples: 1. MBI Fermentas 1 kb DNA ladder; 2. Qbx33 VLP untreated; 3. Qbx33 VLP treated with RNase A; 4. Qbx33 VLP treated with RNase A, packaged with dsCyCpG-253 and treated with DNaseI; 5. Qbx33 VLP treated with RNase A, packaged with dsCyCpG-253, treated with DNaseI and dialysed.

#### *Example 14*

#### *Qβ Disassembly Reassembly and Packaging of Immunostimulatory Nucleic Acids.*

##### *Disassembly and Reassembly of Qβ VLP*

*Disassembly:* 70 mg of pure lyophilized Qβ VLP gave a protein content of about 35 mg, according to spectrophotometric determination using the average result obtained with the following three formulae: 1.  $(183 * OD^{230\text{ nm}} - 75.8 * OD^{260\text{ nm}}) * \text{volume (ml)}$  - 2.  $((OD^{235\text{ nm}} - OD^{280\text{ nm}})/2.51) * \text{volume}$  - 3.  $((OD^{228.5\text{ nm}} - OD^{234.5\text{ nm}}) * 0.37) * \text{volume}$ . The pure lyophilized

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Q $\beta$  VLP was solubilized in 7 ml of 6 M GuHCl and incubated overnight at 4°C. The solution was clarified for 15 minutes, at 6000 rpm (Eppendorf 5810 R, in fixed angle rotor F34-6-38, used in all the following steps). A negligible sediment was discarded, and the supernatant was dialysed 5 X against 200-300 ml NET buffer (20 mM Tris-HCl, pH 7.8 with 5mM EDTA and 150 mM NaCl) over 3 days. Alternatively, the supernatant was dialyzed in a continuous mode against 1.5 l NET buffer over 3-4 days. The resulting suspension was centrifuged at 12000 rpm for 20 minutes. The pellet was resolubilized in 2-3 ml 8 M urea, while the supernatant was precipitated with solid ammonium sulphate at 60% saturation. A saturated ammonium sulphate solution was added to the pellet previously resolubilized in urea to 60% saturation, and the solution was left to precipitate 4 days at 4°C, with subsequent centrifugation at 12000 rpm for 20 minutes. This pellet, and the pellet of the initial supernatant were resolubilized and joined in a total volume of 3 ml of 7 M urea, 10 mM DTT. This material was loaded on a Sephadex G75 column, eluted at 2 ml/h with 7 M urea, 10 mM DTT. Two peaks were isolated. A high molecular weight peak preceded a peak of lower apparent molecular weight. Calibration of the column with chymotrypsin in the same elution buffer revealed that the apparent molecular weight of the second peak is consistent with Q $\beta$  coat protein being in a dimeric form. Fractions containing this dimer material were pooled and precipitated with ammonium sulphate (2 days, at 4°C). The pellet was washed with a few droplets of water, centrifuged again, and solubilized in 2 ml of 7 M urea, 10 mM DTT. This material was then purified on a short (1.5 X 27 cm) Sepharose 4B column. One peak eluted from the column and the fractions were pooled, leading to a protein preparation with a volume of 10 ml, and a ratio of absorbance at 280 nm vs. 260 nm of 0.68/0.5, yielding about 450 nmol of Q $\beta$  coat protein (giving a maximum of 2.5 nmol VLP after reassembly, considering that there are 180 subunits in the VLP) and a protein concentration of 630  $\mu$ g/ml (calculated using the spectrophotometric methods described above).



*Reassembly:*  $\beta$ -mercaptoethanol was added to the 10 ml dimer fraction to a final concentration of 10%, and 300  $\mu$ l of a solution of (CpG)<sub>20</sub>OpA oligodeoxynucleotide, containing 12.3 nmol of oligonucleotide, were added. The reassembly mixture was first dialyzed against 30 ml NET buffer containing 10% beta-mercaptoethanol for 2 hours at 4°C, and then dialyzed in a continuous mode, with a flow of NET buffer of 8 ml/h over 4 days at 4°C. The reassembly mixture was then desalted against water by dialysis, with 6 buffer exchanges (4 X 100 ml, 2 X 1 liter).

The ratio of absorbance at 280 nm vs. 260 nm was of 0.167/0.24. The protein was dried by lyophilization. The dried protein was resolubilized in water and purified by ultracentrifugation on a sucrose gradient in a Beckman L 8-80 centrifuge, with the SW 50.1 rotor at 22 000 rpm, for 17h at +4°C. The sucrose gradient purification was performed as follows. 5 layers of 1 ml of the following sucrose concentrations (w/v) were dispensed into a centrifuge tube: 50%, 43%, 36%, 29% and 22%. The so formed succession of layers was left standing overnight at 4°C. 0.5 ml of the protein sample was layered on the gradient, and centrifuged for 17 h as indicated above. The gradient was eluted from the bottom of the centrifuge tube, and the 5 ml of the gradient were divided in 16 fractions of approximatively 300  $\mu$ l. The fractions in the gradient were analyzed by SDS-PAGE (Figure 28) and Ouchterlony assay. Fractions 6-9 contained Q $\beta$  coat protein and gave the precipitation band typical of Q $\beta$  VLP in an Ouchterlony assay. Fractions 11-15, with a lower apparent density and containing Q $\beta$  protein gave no capsid band in the Ouchterlony assay. The reassembled Q $\beta$  had the same apparent density as wt Q $\beta$  within experimental error. The fractions 6-9 of the sucrose gradient were pooled, dialyzed against water and lyophilized. This material was then resolubilized for electron microscopy (EM) analysis (Figure 29) and Ouchterlony assay (Figure 30 A and B). The EM procedure was as follows: A suspension of the proteins was absorbed on carbon-formvar coated grids and stained with 2% phosphotungstic acid (pH 6,8). The grids were examined with a JEM 100C

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(JEOL, Japan) electron microscope at an accelerating voltage of 80 kV. Photographic records (negatives) were performed on Kodak electron image film and electron micrographs were obtained by printing of negatives on Kodak Polymax paper. Both methods indicate that the reassembled VLPs have the same macromolecular properties as intact Q $\beta$  VLP. In addition, the pattern of disulfide bonds displayed by the purified reassembled Q $\beta$  VLP is indistinguishable from the disulfide bond pattern displayed by the untreated Q $\beta$  VLP, with the typical pattern of pentamers and hexamers (Figure 31 A).

*Analysis of nucleic acid content:* Reassembled Q $\beta$  VLP was digested with pancreatic DNase I as follows. To 200  $\mu$ l of a 0.5 mg/ml solution of Q $\beta$  VLP reassembled with (CpG)<sub>20</sub>OpA oligodeoxynucleotide were added 20  $\mu$ l of a 1 U/ $\mu$ l DNase I (Fluka) solution, and 22  $\mu$ l of DNase I buffer (20 mM MgCl<sub>2</sub>, 200 mM Tris, pH 8.3). The reaction mixture was incubated for 2h30 min. at 37°C. The nucleic acid content of the sample was subsequently isolated by phenol/chloroform extraction, and loaded on a 2 % agarose gel stained with ethidium bromide (Figure 31B). A band of the size of the packaged oligodeoxynucleotide was detected on the gel. A band migrating at a higher apparent molecular weight was also visible. We cannot exclude the presence of multimers of the (CpG)<sub>20</sub>OpA oligodeoxynucleotide which would lead to a band at this height. The gel thus shows that DNase I protected oligodeoxynucleotides of the right size were present in the reassembled Q $\beta$  VLP, since the oligodeoxynucleotides could subsequently be digested by DNase I, but not by RNase A. Oligodeoxynucleotides could thus be successfully packaged in Q $\beta$  VLP after initial disassembly of the VLP, purification of the disassembled coat protein from nucleic acids and subsequent reassembly of the VLP in the presence of oligodeoxynucleotide.

Figure 28 shows the SDS-PAGE analysis of the fractions from the sucrose gradient centrifugation. Loaded on the gels were the following samples. Lane 1 – 10: fractions 6-15 of the sucrose gradient ultracentrifugation.

Figure 29 shows the EM pictures of (A) intact Q $\beta$  VLP and (B) Q $\beta$  VLP after disassembly and reassembly in the presence of oligonucleotide (CpG)<sub>20</sub>OpA, and subsequent purification by sucrose gradient ultracentrifugation. A dense overlay of capsids is observed, and those capsids display the same structural features and properties as the intact Q $\beta$  VLPs.

Figure 30 shows the Ouchterlony analysis (immunodiffusion) of the reconstructed Q $\beta$  VLP. In Figure 30 A, Q $\beta$  VLP reassembled with oligonucleotide (CpG)<sub>20</sub>OpA was loaded next to intact Q $\beta$  VLP. The two characteristic precipitation bands are indicated by black arrows. The two precipitation bands are concurrent, indicating that the reassembled Q $\beta$  VLP diffuse as the intact Q $\beta$  VLP. In Figure 30 B, sample 1 is Q $\beta$  VLP reassembled in the presence of ribosomal RNA, while sample 2 is intact Q $\beta$  VLP and sample 3 is Q $\beta$  VLP reassembled with oligonucleotide (CpG)<sub>20</sub>OpA. The precipitation bands are indicated by white arrows.

Figure 31 A shows the analysis of the untreated and reassembled Q $\beta$  VLP by non-reducing SDS-PAGE. The pentamers and hexamers of Q $\beta$  VLP are indicated by arrows.

Figure 31 B shows the agarose gel electrophoresis analysis of the nucleotide content extracted after DNase I digestion of Q $\beta$  VLP reassembled with oligonucleotide (CpG)<sub>20</sub>OpA. The nucleic acid content was either untreated (lane 1), or subsequently digested with DNase I (lane2) or RNase A (lane 3); 33  $\mu$ g of reassembled Q $\beta$  VLP were loaded on each lane. 300 ng of a 50 bp oligonucleotide were loaded on lane 4, while 10  $\mu$ l of the GeneRuler 100 bp DNA ladder + marker (MBI Fermentas) was loaded on lane 5.

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**Example 15**

***Q $\beta$  Disassembly Reassembly with different immunostimulatory nucleic acids.***

***Disassembly and Reassembly of Q $\beta$  VLP with oligodeoxynucleotides of various sequences***

The disassembly of Q $\beta$  VLP was performed essentially as described in Example 1, but for the use of 8 M urea instead of 7 M urea to resuspend the ammonium sulphate pellets.

The reassembly of Q $\beta$  VLP with the oligos CyOpA, CyCyCy, (CpG)<sub>20</sub>-OpA and CyCpG was performed essentially as described in Example 1, but for the following variations. A dialysis step against 10 %  $\beta$ -mercaptoethanol in NET buffer (20 mM Tris-HCl, pH 7.8 with 5mM EDTA and 150 mM NaCl) for 1 hour at 4 °C was added to the procedure before addition of the oligodeoxynucleotide solution to the dimer solution in the dialysis bag. The oligodeoxynucleotides were then added to the dimer solutions, resulting approx. in a ten-fold molar excess of oligonucleotide to capsid (180 subunits) as described previously. The reassembly mixture was first dialyzed against 30 ml NET buffer containing 10%  $\beta$ -mercaptoethanol for 1 hours at 4°C, and then dialyzed in a continuous mode, with a flow of NET buffer of 8 ml/h over 4 days at 4°C. A sample of the reassembly reaction of Q $\beta$  VLP with oligodeoxynucleotide CyOpA was taken for EM analysis (Figure 32) at the end of the reassembly reaction. The EM procedure using phosphotungstic acid and described above was used. The reassembly mixtures were then desalted against water by dialysis and dried.

The dried protein was resolubilized in water and purified by ultracentrifugation on a sucrose gradient. The purified reassembled Q $\beta$  VLPs were also analyzed by EM (Figure 33 A-D). The electron micrographs indicate that the reassembled VLPs have the same macromolecular properties as intact Q $\beta$  VLP. Purification notably enriches the preparations for reassembled

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particles. Thus, Q $\beta$  VLP was successfully reassembled with oligodeoxynucleotides of various lengths and sequences.

*Coupling of the p33 peptide to reassembled Q $\beta$  VLP:* Q $\beta$  VLP reassembled with the oligodeoxynucleotide CyOpA was reacted at a concentration of 1.5 mg/ml, with the cross-linker SMPH diluted from a stock solution in DMSO at a final concentration of cross-linker of 536  $\mu$ M for 35 minutes at 26°C in 20 mM Hepes pH 7.4. The derivatized Q $\beta$  VLP was dialyzed 2 X 2 hours against a thousand volumes of 20 mM Hepes, 150 mM NaCl, pH 7.4. The dialysed derivatized Q $\beta$  VLP at a concentration of 1.4 mg/ml was subsequently reacted with the p33GGC peptide (sequence: KAVYNFATMGGC) at a final concentration of peptide of 250  $\mu$ M for 2 hours at 15°C in 20 mM Hepes, 150 mM NaCl, pH 7.4. The gel of Figure 34 indicates successful coupling of the p33 peptide to Q $\beta$  VLP. Coupling bands corresponding to one, respectively two peptides coupled per subunit are indicated by an arrow in the Figure.

*Analysis of nucleic acid content:* The nucleic acid content of reassembled and coupled Q $\beta$  VLP was analysed by proteinase K digestion, phenol chloroform extraction and subsequent loading of the extracted oligonucleotide on a TBE/Urea PAGE gel. The analysis procedure was as follows. 25  $\mu$ l reassembled Q $\beta$  VLP (0.5-1 mg/ml) were supplemented with 0.5  $\mu$ l proteinase K, 1.5  $\mu$ l 10% SDS and 3  $\mu$ l 10x proteinase buffer (0.5 M NaCl, 50 mM EDTA, 0.1 M Tris pH7.4). After incubation overnight at 37°C, proteinase K was inactivated by heating 20 min at 65°C and the nucleic acid content was extracted from the samples by 1x phenol and 1x chloroform extraction. Subsequently the samples were incubated 2 h at 37°C with 1  $\mu$ l RNaseA (Qiagen, 100  $\mu$ g/ml, diluted 250x). The equivalent of 2  $\mu$ g starting protein was heated 3 min at 95°C with 1 volume of 2x loading buffer (1ml 10xTBE, 4.2 g Urea, 1.2 g Ficoll, 1 ml 0.1%Bromophenolblue, H<sub>2</sub>O up to 10 ml) and loaded on a 15% TBE/Urea polyacrylamide gel (Invitrogen). The gel was run for 1.5 h at 180 V, and fixed in 10% acetic acid/20% ethanol and stained with CYBR Gold (Molecular Probes, Eugene ,OR, USA). For

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quantification, 10 and 20 pmol of the oligonucleotide used for the reassembly were applied on the gel as a reference. Resistance of the nucleic acid content to RNase and its size proved that the packaged nucleic acid was the oligonucleotide used for reassembly. Quantification of the packaged oligodeoxynucleotide was performed by comparison of the band intensity of the extracted oligonucleotide with the band intensity of a reference amount of the same oligonucleotide loaded on the same gel. A figure of 1.75 nmol CyOpA/ 100  $\mu$ g Q $\beta$  VLP was obtained, giving a ratio of 44 oligonucleotides per VLP on average.

Figure 32 depicts the electron micrographs of the reassembly reaction of Q $\beta$  VLP with the oligonucleotide CyOpA before purification. The magnification was 200 000 fold.

Figure 33 A-D show the electron micrographs of the purified reassembly reactions of Q $\beta$  VLP with the oligodeoxynucleotides Cy(CpG)20 (A), CyCyCy (B), CyCpG (C) and CyOpA (D). The magnification was 200 000 fold.

Figure 34 depicts the SDS-PAGE analysis of the coupling of Q $\beta$  VLP reassembled with the oligodeoxynucleotide CyOpA to the p33GGC peptide. Loaded on the gel were the following samples: 1. Prestained Protein Marker, Broad Range (# 7708S) 10  $\mu$ l; 2. Q $\beta$  VLP reassembled with CyOpA [1.5 mg/ml] 10  $\mu$ l; 3. Q $\beta$  VLP reassembled with CyOpA [1.5 mg/ml] and derivatized with SMPH 10  $\mu$ l; 4. Q $\beta$  VLP reassembled with CyOpA [1.5 mg/ml], derivatized with SMPH and coupled with p33-peptide 10  $\mu$ l; 5. Q $\beta$  VLP reassembled with CyOpA [1.5 mg/ml], derivatized with SMPH and coupled with p33-peptide, 1/5<sup>th</sup> vol of the pellet.

Figure 35 depicts the analysis of the extracted packaged oligodeoxynucleotides by Urea Polyacrylamide gel electrophoresis, stained with CYBR Gold. The following samples were loaded on the gel: 1. Q $\beta$  VLP reassembled with oligonucleotide CyOpA and coupled to p33 GGC peptide, 2  $\mu$ g protein loaded on the gel. 2. Q $\beta$  VLP reassembled with oligonucleotide CyOpA and coupled to p33GGC peptide, frozen and thawed before loading on

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the gel, 2  $\mu$ g protein. 3. Q $\beta$  VLP reassembled with oligonucleotide Cy(CpG)<sub>20</sub> and coupled to p33GGC peptide, frozen and thawed before loading on the gel; 2  $\mu$ g protein. 4. CyOpA oligonucleotide, 20 pmol. 5. CyOpA oligonucleotide, 10 pmol.

### ***Example 16***

#### ***Q $\beta$ Disassembly Reassembly and Packaging.***

##### *Disassembly and Reassembly of Q $\beta$ VLP*

*Disassembly:* 10 mg Q $\beta$  VLP (as determined by Bradford analysis) in 20 mM HEPES, pH 7.4, 150 mM NaCl was precipitated with solid ammonium sulfate at a final saturation of 60%. Precipitation was performed over night at 4°C and precipitated VLPs were sedimented by centrifugation for 60 minutes at 4°C (SS-34 rotor). Pellets were resuspended in 1 ml of 6 M Guanidine hydrochloride (GuHCl) containing 100 mM DTT (final concentration) and incubated for 8 h at 4°C.

*Purification of Q $\beta$  coat protein by size exclusion chromatography:* The solution was clarified for 10 minutes at 14000 rpm (Eppendorf 5417 R, in fixed angle rotor F45-30-11, used in all the following steps) and dialysed against a buffer containing 7 M urea, 100 mM TrisHCl, pH 8.0, 10 mM DTT (2000 ml) over night. Dialysis buffer was exchanged once and dialysis continued for another 2 h. The resulting suspension was centrifuged at 14 000 rpm for 10 minutes at 4°C. A negligible sediment was discarded, and the supernatant was kept as "load fraction" containing disassembled coat protein and RNA. Protein concentration was determined by Bradford analysis and 5mg total protein was applied onto a HiLoad™ Superdex™ 75 prep grade column (26/60, Amersham Biosciences) equilibrated with 7 M urea, 100 mM TrisHCl and 10 mM DTT. Size exclusion chromatography was performed with the equilibration buffer (7 M urea, 100 mM TrisHCl pH 8.0, 10 mM DTT) at 12°C with a flow-rate of 0.5 ml/min. During the elution absorbance

at 254 nm and 280 nm was monitored. Two peaks were isolated. A high molecular weight peak preceded a peak of lower apparent molecular weight. Peaks were collected in fractions of 1.5 ml and aliquots were analysed by SDS-PAGE followed by Coomassie staining as well as SYBR®Gold staining (Figure 36).

*Purification of Q $\beta$  coat protein by ion exchange chromatography:* Alternatively, the clarified supernatant was dialysed against a buffer containing 7 M urea, 20 mM MES, 10 mM DTT, pH 6.0 (2000 ml) over night. Dialysis buffer was exchanged once and dialysis continued for another 2 h. The resulting suspension was centrifuged at 14 000 rpm for 10 minutes at 4°C. A negligible sediment was discarded, and the supernatant was kept as “load fraction” containing disassembled coat protein and RNA. Protein concentration was determined by Bradford analysis and 10 mg total protein was diluted to a final volume of 10 ml with buffer A (see below) and applied with a flowrate of 1 ml/min to a 1 ml HiTrap™ SP HP column (Amersham Biosciences, Cat. No. 17-1151-01 ) equilibrated with buffer A: 7 M urea, 20 mM MES, 10 mM DTT, pH 6.0. The flowthrough which contained the RNA was collected as one fraction. After the column was extensively washed with buffer A (30 CV) the bound Q $\beta$  coat protein was eluted in a linear gradient from 0% - 100% buffer B (gradient length was 5 CV; buffer A: see above, buffer B: 7 M urea, 20 mM MES, 10 mM DTT, 2 M NaCl, pH 6.0). During the loading, wash and elution the absorbance at 254 nm and 280 nm was monitored. Peak fractions of 1 ml were collected and analysed by SDS-PAGE followed by Coomassie staining as well as SYBR®Gold staining. Fractions containing the Q $\beta$  coat protein but not the RNA were identified and the pH was adjusted by addition of 100  $\mu$ l 1 M TrisHCl, pH 8.0.

Samples containing the Q $\beta$  coat protein but no RNA were pooled and dialysed against 0.87 M urea, 100 mM TrisHCl, 10 mM DTT (2000 ml) over night and buffer was exchanged once and dialysis continued for another 2 h. The resulting suspension was centrifuged at 14 000 rpm for 10 minutes at 4°C. A negligible sediment was discarded, and the supernatant was kept as



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“disassembled coat protein”. Protein concentration was determined by Bradford analysis.

*Reassembly:* Purified Q $\beta$  coat protein with a concentration of 0.5 mg/ml was used for the reassembly of VLPs in the presence of an oligodeoxynucleotide. For the reassembly reaction the oligodeoxynucleotide was used in a tenfold excess over the calculated theoretical amount of Q $\beta$ -VLP capsids (monomer concentration divided by 180). After the Q $\beta$  coat protein was mixed with the oligodeoxynucleotide to be packaged during the reassembly reaction, this solution (volume up to 5 ml) was first dialysed for 2 h against 500 ml NET buffer containing 10%  $\beta$ -mercaptoethanol at 4°C, then dialyzed in a continuous mode, with a flow of NET buffer of 8 ml/h over 72 h at 4°C, and finally for another 72 h with the same continuous mode with a buffer composed of 20 mM TrisHCl pH 8.0, 150 mM NaCl. The resulting suspension was centrifuged at 14 000 rpm for 10 minutes at 4°C. A negligible sediment was discarded, and the supernatant contained the reassembled and packaged VLPs. Protein concentration was determined by Bradford analysis and if needed reassembled and packaged VLPs were concentrated with centrifugal filter devices (Millipore, UFV4BCC25, 5K NMWL) to a final protein concentration of 3 mg/ml.

*Purification of reassembled and packaged VLPs:* Up to 10 mg total protein was loaded onto a Sepharose™ CL-4B column (16/70, Amersham Biosciences) equilibrated with 20 mM HEPES pH 7.4, 150 mM NaCl. Size exclusion chromatography was performed with the equilibration buffer (20 mM HEPES pH 7.4, 150 mM NaCl) at room temperature with a flow-rate of 0.4 ml/min. During the elution absorbance at 254 nm and 280 nm was monitored. Two peaks were isolated. A high molecular weight peak preceded a peak of lower apparent molecular weight. Fractions of 0.5 ml were collected and analysed by SDS-PAGE followed by Coomassie blue staining (Figure 37). Calibration of the column with intact and highly purified Q $\beta$  capsids from *E.coli* revealed that the apparent molecular weight of the major first peak was consistent with Q $\beta$  capsids.

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Analysis of Q $\beta$  VLPs which had been reassembled in the presence of oligodeoxynucleotides:

- A) *Overall structure of the capsids:* Q $\beta$  VLPs that were reassembled either in the presence of one of the following oligodeoxynucleotides (CyOpA, Cy(CpG)20OpA, Cy(CpG)20, CyCyCy, (CpG)20OpA), or in the presence of tRNA from *E.coli* (Roche Molecular Biochemicals, Cat. No. 109541) were analyzed by electron microscopy (negative staining with uranylacetate pH 4.5) and compared to intact Q $\beta$  VLPs purified from *E.coli*. As a negative control served a reassembly reaction where nucleic acid was omitted. Reassembled capsids display the same structural features and properties as the intact Q $\beta$  VLPs (Figure 38).
- B) *Hydrodynamic size of reassembled capsids:* Q $\beta$  capsids which had been reassembled in the presence of oligodeoxynucleotides were analyzed by dynamic light scattering (DLS) and compared to intact Q $\beta$  VLPs which had been purified from *E.coli*. Reassembled capsids showed the same hydrodynamic size (which depends both on mass and conformation) as the intact Q $\beta$  VLPs.
- C) *Disulfide-bond formation in reassembled capsids:* Reassembled Q $\beta$  VLPs were analyzed by native polyacrylamid gelelectrophoresis and compared to intact Q $\beta$  VLPs which had been purified from *E.coli*. Reassembled capsids displayed the same disulfide-bond pattern as the intact Q $\beta$  VLPs (Figure 39).
- D) *Analysis of nucleic acid content of the Q $\beta$  VLPs which had been reassembled in the presence of oligodeoxynucleotides by agarose gelelectrophoresis:* 5  $\mu$ g reassembled Q $\beta$  VLPs were incubated in total reaction volume of 25  $\mu$ l either with 0.35 units RNase A (Qiagen, Cat. No. 19101), 15 units DNase I (Fluka, Cat. No. 31136), or without any further addition of enzymes for 3 h at 37°C. Intact Q $\beta$  VLPs which had been purified from *E.coli* served as control and were incubated under the same conditions as described for the capsids which had been

reassembled in the presence of oligodeoxynucleotides. The reactions were then loaded on a 0.8% agarose gel that was first stained with ethidiumbromide (Figure 40A) and subsequently with Coomassie blue (Figure 40B). The ethidium bromide stain shows, that none of the added enzymes could digest the nucleic acid content in the reassembled Q $\beta$  capsids showing that the nucleic acid content (i.e. the oligodeoxynucleotides) is protected. This result indicates that the added oligodeoxynucleotides were packaged into the newly formed capsids during the reassembly reaction. In contrast, the nucleic acid content in the intact Q $\beta$  VLPs which had been purified from *E.coli* was degraded upon addition of RNase A, indicating that the nucleic acid content in this VLPs consists of RNA. In addition, both the ethidium bromide stain and the Coomassie blue stain of the agarose gel shows that the nucleic acid containing Q $\beta$  VLPs (reassembled and purified from *E.coli*, respectively) are migrating at about the same size, which indicates that the reassembly reaction led to Q $\beta$  VLPs of comparable size to intact Q $\beta$  VLPs which had been purified from *E.coli*.

The gel thus shows that DNase I protected oligodeoxynucleotides were present in the reassembled Q $\beta$  VLP. Furthermore, after the packaged oligodeoxynucleotides had been extracted by phenol/chloroform they were digestable by DNase I, but not by RNase A. Oligodeoxynucleotides could thus be successfully packaged into Q $\beta$  VLPs after initial disassembly of the VLP, purification of the disassembled coat protein from nucleic acids and subsequent reassembly of the VLPs in the presence of oligodeoxynucleotides.

- E) *Analysis of nucleic acid content of the Q $\beta$  VLPs which had been reassembled in the presence of oligodeoxynucleotides by denaturing polyacrylamide TBE-Urea gelelectrophoresis:* 40  $\mu$ g reassembled Q $\beta$  VLPs (0.8 mg/ml) were incubated in a total reaction volume of 60  $\mu$ l with 0.5 mg/ml proteinase K (PCR-grade, Roche Molecular

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Biochemicals, Cat. No. 1964364) and a reaction buffer according to the manufacturers instructions for 3 h at 37°C. Intact Q $\beta$  VLPs which had been purified from *E.coli* served as control and were incubated with proteinase K under the same conditions as described for the capsids which had been reassembled in the presence of oligodeoxynucleotides. The reactions were then mixed with a TBE-Urea sample buffer and loaded on a 15% polyacrylamide TBE-Urea gel (Novex<sup>®</sup>, Invitrogen Cat. No. EC6885). As a qualitative as well as quantitative standard, 1 pmol, 5 pmol and 10 pmol of the oligodeoxynucleotide which was used for the reassembling reaction, were loaded onto the same gel. This gel was fixed with 10% acetic acid, 20% methanol, equilibrated to neutral pH and stained with SYBR<sup>®</sup>-Gold (Molecular Probes Cat. No. S-11494). The SYBR<sup>®</sup>-Gold stain showed (Figure 41), that the reassembled Q $\beta$  capsids contained nucleic acid comigrating with the oligodeoxynucleotides which were used in the reassembly reaction. Note that intact Q $\beta$  VLPs (which had been purified from *E.coli*) did not contain a nucleic acid of similar size. Taken together, analysis of the nucleic acid content of the Q $\beta$  VLPs which had been reassembled in the presence of oligodeoxynucleotides showed that oligodeoxynucleotides were protected from DNase I digestion, meaning that they were packaged) and that the added oligodeoxynucleotides could be reisolated by proper means (e.g. proteinase K digestion of the Q $\beta$  VLP).

Figure 36 shows the purification of disassembled Q $\beta$  coat protein by size exclusion chromatography. 5  $\mu$ l of the indicated fractions (#) were mixed with sample buffer and loaded onto 16% Tris-Glycine gels (Novex<sup>®</sup> by Invitrogen, Cat. No. EC64952). After the run was completed the gels were stained first with Coomassie blue (A) and after documentation the same gels were stained with SYBR<sup>®</sup>-Gold (B). Note that the first high molecular weight peak (fractions #15 - #20) contained no protein but nucleic acids. On the other

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hand, the second peak of lower apparent molecular weight contained disassembled coat protein which was thereby separated from the nucleic acids.

Figure 37 shows the purification of reassembled Q $\beta$  VLPs by size exclusion chromatography.

10  $\mu$ l of the indicated fractions (#) were mixed with sample buffer and loaded onto a 16% Tris-Glycine gel (Novex<sup>®</sup> by Invitrogen, Cat. No. EC64952). After the run was completed the gel was stained with Coomassie blue. Due to the reducing conditions, disulfide bonds were reduced and the proteinaceous monomer of the reassembled VLPs is visible as 14 kDa coat protein.

Figure 38 shows electron micrographs of Q $\beta$  VLPs that were reassembled in the presence of different oligodeoxynucleotides. The VLPs had been reassembled in the presence of the indicated oligodeoxynucleotides or in the presence of tRNA but had not been purified to a homogenous suspension by size exclusion chromatography. As positive control served preparation of "intact" Q $\beta$  VLPs which had been purified from *E.coli*. Importantly, by adding any of the indicated nucleic acids during the reassembly reaction, VLPs of the correct size and conformation could be formed, when compared to the "positive" control. This implicates that the reassembly process in general is independent of the nucleotide sequence and the length of the used oligodeoxynucleotides. Note that adding of nucleic acids during the reassembly reaction is required for the formation of Q $\beta$  VLPs, since no particles had been formed if nucleic acids were omitted from the reassembly reaction.

Figure 39 shows the analysis of the disulfide-bond pattern in reassembled and purified Q $\beta$  capsids. 5  $\mu$ g of the indicated capsids were mixed with sample buffer that either contained a reducing agent or not and loaded onto a 16% Tris-Glycine gel. After the run was completed the gel was stained with Coomassie blue. When compared to "intact" capsids purified from *E.coli*, the reassembled Q $\beta$  VLPs displayed the same disulfide bond pattern.

Figure 40 shows the analysis of nucleic acid content of the reassembled Q $\beta$  VLPs by nuclease treatment and agarose gelelectrophoresis: 5

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$\mu\text{g}$  of reassembled and purified Q $\beta$  VLPs and 5  $\mu\text{g}$  of Q $\beta$  VLPs which had been purified from *E.coli*, respectively, were treated as indicated. After this treatment, samples were mixed with loading dye and loaded onto a 0.8% agarose gel. After the run the gel was stained first with ethidium bromide (A) and after documentation the same gel was stained with Coomassie blue (B). Note that the nucleic acid content of the reassembled and purified Q $\beta$  VLPs were resistant towards RNase A digestion while the nucleic acid content of Q $\beta$  VLPs purified from *E.coli* was digested upon incubation with RNase A. This indicates that the nucleic acid content of the reassembled Q $\beta$  capsids consists out of deoxynucleotides which of course are protected from RNase A digestion. Hence, oligodeoxynucleotides were packaged into Q $\beta$  VLPs during the reassembly reaction.

Figure 41 shows the analysis of nucleic acid content of the reassembled Q $\beta$  VLPs by proteinase K treatment and polyacrylamide TBE/Urea gelelectrophoresis: The equivalent of 1  $\mu\text{g}$  Q $\beta$  VLPs which had been digested by proteinase K-treatment was mixed with a TBE-Urea sample buffer and loaded on a 15% polyacrylamide TBE-Urea gel (Novex<sup>®</sup>, Invitrogen Cat. No. EC6885). As qualitative as well as quantitative standard, 1 pmol, 5 pmol and 10 pmol of the oligodeoxynucleotide which was used for the reassembly reaction, was loaded onto the same gel. After the run was completed, the gel was fixed, equilibrated to neutral pH and stained with SYBR<sup>®</sup>-Gold (Molecular Probes Cat. No. S-11494). Note that intact Q $\beta$  VLPs (which had been purified from *E.coli*) did not contain nucleic acids of similar size than those which had been extracted from reassembled Q $\beta$  capsids. In addition, nucleic acids isolated from reassembled VLPs were comigrating with the oligodeoxynucleotides which had been used in the reassembly reaction. This results confirmed that the used oligodeoxynucleotides were packaged into reassembled Q $\beta$  capsids.

**Example 17*****AP205 Disassembly-Purification-Reassembly and Packaging of immunostimulatory nucleic acids.******A. Disassembly and Reassembly of AP205 VLP from material able to reassemble without addition of oligonucleotide***

*Disassembly:* 40 mg of lyophilized purified AP205 VLP were resolubilized in 4 ml 6 M GuHCl, and incubated overnight at 4°C. The disassembly mixture was centrifuged at 8000 rpm (Eppendorf 5810 R, in fixed angle rotor F34-6-38, used in all the following steps). The pellet was resolubilized in 7 M urea, while the supernatant was dialyzed 3 days against NET buffer (20 mM Tris-HCl, pH 7.8 with 5mM EDTA and 150 mM NaCl) with 3 changes of buffer. Alternatively, dialysis was conducted in continuous mode over 4 days. The dialyzed solution was centrifuged at 8000 rpm for 20 minutes, and the pellet was resolubilized in 7 M urea, while the supernatant was pelleted with ammonium sulphate (60% saturation), and resolubilized in a 7 M urea buffer containing 10 mM DTT. The previous pellets all resolubilized in 7 M urea were joined, and precipitated with ammonium sulphate (60% saturation), and resolubilized in a 7 M urea buffer containing 10 mM DTT. The materials resolubilized in the 7 M urea buffer containing 10 mM DTT were joined and loaded on a Sephadex G75 column equilibrated and eluted with the 7 M urea buffer containing 10 mM DTT at 2ml/h. One peak eluted from the column. Fractions of 3 ml were collected. The peak fractions containing AP205 coat protein were pooled and precipitated with ammonium sulphate (60% saturation). The pellet was isolated by centrifugation at 8000 rpm, for 20 minutes. It was resolubilized in 7 M urea, 10 mM DTT, and loaded on a short Sepharose 4B column (1.5 X 27 cm Sepharose 4B, 2 ml/h, 7 M urea, 10 mM DTT as elution buffer). Mainly one peak, with a small shoulder eluted from the column. The fractions containing the AP205 coat protein were identified by SDS-PAGE, and pooled, excluding the shoulder.

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This yielded a sample of 10.3 ml. The protein concentration was estimated spectrophotometrically by measuring an aliquot of protein diluted 25-fold for the measurement, using the following formula:  $(1.55 \times \text{OD}_{280} - 0.76 \times \text{OD}_{260}) \times \text{volume}$ . The average concentration was of 1 nmol/ml of VLP (2.6 mg/ml). The ratio of absorbance at 280 nm vs. 260 nm was of 0.12/0.105.

*Reassembly:* 1.1 ml beta-mercaptoethanol was added to the sample, and the following reassembly reactions were set up:

1. 1 ml of AP205 coat protein, no nucleic acids
2. 1 ml of AP205 coat protein, rRNA (approx. 200 OD260 units, 10 nmol)
3. 9 ml of AP205 coat protein, CyCpG (370  $\mu\text{l}$  of 225 pmol/ $\mu\text{l}$  solution, i.e. 83 nmol).

These mixtures were dialyzed 1 hour against 30 ml of NET buffer containing 10% beta-mercaptoethanol. The mixture containing no nucleic acids was dialyzed separately. The dialysis was then pursued in a continuous mode, and 1 l of NET buffer was exchanged over 3 days. The reaction mixtures were subsequently extensively dialyzed against water (5 changes of buffer), and lyophilized. They were resolubilized in water, and analyzed by EM. All mixtures contained capsids, showing that AP205 VLP reassembly is independent of the presence of detectable nucleic acids, as measured by agarose gel electrophoresis using ethidium bromide staining. The EM analysis of AP205 reassembled with CyCpG is shown on Figure 42B. The EM procedure was as follows: A suspension of the proteins was absorbed on carbon-formvar coated grids and stained with 2% phosphotungstic acid (pH 6,8). The grids were examined with a JEM 100C (JEOL, Japan) electron microscope at an accelerating voltage of 80 kV. Photographic records (negatives) were performed on Kodak electron image film and electron micrographs were obtained by printing of negatives on Kodak Polymax paper. The VLP reassembled in the presence of the CyCpG was purified over a Sepharose 4B column (1 X 50 cm), eluted with NET buffer (1 ml/h). The fractions were analyzed by Ouchterlony assay, and the fractions containing



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VLP were pooled. This resulted in a sample of 8 ml, which was desalted against water by dialysis, and dried. The yield of capsid was of 10 mg. Analysis of resolubilized material in a 0.6% agarose gel stained with ethidium-bromide showed that the capsids were empty of nucleic acids. Samples of the reassembly reaction containing CyCpG taken after the reassembly step and before extensive dialysis were analysed on a 0.6% agarose gel and are shown in Figure 43A and B. A band migrating at the same height than intact AP205 VLP and staining both for ethidium-bromide and Coomassie blue staining could be obtained, showing that AP205 VLP containing oligodeoxynucleotide had been reassembled. The extensive dialysis steps following the reassembly procedure are likely to have led to diffusion of the oligodeoxynucleotide outside of the VLPs. Significantly, the AP205 VLPs could also be reassembled in the absence of detectable oligodeoxynucleotide, as measured by agarose gel electrophoresis using ethidium bromide staining. Oligodeoxynucleotides could thus be successfully bound to AP205 VLP after initial disassembly of the VLP, purification of the disassembled coat protein from nucleic acids and subsequent reassembly of the VLP in the presence of oligodeoxynucleotide.

Figure 42 shows electron micrographs of either intact recombinant AP205 VLP used for the disassembly step (A), or AP205 VLP disassembled, and subsequently reassembled in the presence of CyCpG (B).

Figure 43 shows the agarose gel-electrophoresis analysis of the AP205 VLP sample reassembled in the presence of CyCpG, and taken directly after the reassembly step before dialysis. The gel on Figure 43A was stained with ethidium-bromide. AP205 VLP reassembled with CyCpG was loaded on lane 1, while untreated pure AP205 VLP was loaded on lane 2. The arrow indicates the band of the reassembled AP205 VLP. The gel on Figure 43 B was stained with Coomassie-brilliant blue. Untreated AP205 VLP was loaded on lane 1, while AP205 VLP reassembled with CyCpG was loaded on on lane 2.

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*B. Reassembly of AP205 VLP using disassembled material which does not reassemble in the absence of added oligonucleotide*

*Disassembly:* 100 mg of purified and dried recombinant AP205 VLP (Cytos patent) were used for disassembly as described above. All steps were performed essentially as described under disassembly in part A, but for the use of 8 M urea to solublize the pellets of the ammonium sulphate precipitation steps and the omission of the gel filtration step using a CL-4B column prior to reassembly. The pooled fractions of the Sephadex G-75 column contained 21 mg of protein as determined by spectroscopy using the formula described in part A. The ratio of absorbance at 280 nm to the absorbance at 260 nm of the sample was of 0.16 to 0.125. The sample was diluted 50 times for the measurement.

*Reassembly:* The protein preparation resulting from the Sephadex G-75 gel filtration purification step was precipitated with ammonium sulphate at 60% saturation, and the resulting pellet solubilized in 2 ml 7 M urea, 10 mM DTT. The sample was diluted with 8 ml of 10% 2-mercaptoethanol in NET buffer, and dialyzed for 1 hour against 40 ml of 10% 2-mercaptoethanol in NET buffer. Reassembly was initiated by adding 0.4 ml of a CyCpG solution (109 nmol/ml) to the protein sample in the dialysis bag. Dialysis in continuous mode was set up, and NET buffer used as eluting buffer. Dialysis was pursued for two days and a sample was taken for EM analysis after completion of this dialysis step (Figure 44 B). The dialyzed reassembly solution was subsequently dialyzed against 50% v/v Glycerol in NET buffer, to achieve concentration. One change of buffer was effected after one day of dialysis. The dialysis was pursued over a total of three days.

The dialyzed and concentrated reassembly solution was purified by gel filtration over a Sepharose 4-B column (1X60 cm) at a flow rate of 1 ml/hour, in NET buffer. Fractions were tested in an Ouchterlony assay, and fractions containing capsids were dried, resuspended in water, and rechromatographed

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on the 4-B column equilibrated in 20 mM Hepes pH 7.6. Using each of the following three formula:

1.  $(183 * OD^{230\text{ nm}} - 75.8 * OD^{260\text{ nm}}) * \text{volume (ml)}$  – 2.  $((OD^{235\text{ nm}} - OD^{280\text{ nm}})/2.51) * \text{volume}$  – 3.  $((OD^{228.5\text{ nm}} - OD^{234.5\text{ nm}}) * 0.37) * \text{volume}$   
protein amounts of 6 – 26 mg of reassembled VLP were determined.

The reassembled AP205 VLPs were analyzed by EM as described above, agarose gel electrophoresis and SDS-PAGE under non-reducing conditions.

The EM analysis of disassembled material shows that the treatment of AP205 VLP with guanidinium-chloride essentially disrupts the capsid assembly of the VLP. Reassembly of this disassembled material with an oligonucleotide yielded capsids (Figure 44B), which were purified and further enriched by gel filtration (Figure 44 C). Two sizes of particles were obtained; particles of about 25 nm diameter and smaller particles are visible in the electron micrograph of Figure 44C. No reassembly was obtained in the absence of oligonucleotides. Loading of the reassembled particles on agarose electrophoresis showed that the reassembled particles contained nucleic acids. Extraction of the nucleic acid content by phenol extraction and subsequent loading on an agarose gel stained with ethidium bromide revealed that the particles contained the oligonucleotide used for reassembly (Figure 45A). Identity of the packaged oligonucleotide was controlled by loading a sample of this oligonucleotide side to side to the nucleic acid material extracted from the particles. The agarose gel where the reassembled AP205 VLP had been loaded and previously stained with ethidium bromide was subsequently stained with Coomassie blue, revealing comigration of the oligonucleotide content with the protein content of the particles (Figure 45B), showing that the oligonucleotide had been packaged in the particles.

Loading of the reassembled AP205 VLP on an SDS-PAGE gel, run in the absence of reducing agent (Figure 46) demonstrated that the reassembled particles have formed disulfide bridges, as is the case for the untreated AP205

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VLP. Moreover, the disulfide bridge pattern is identical to the untreated particles.

Depicted on Figure 44 A is an electron micrograph of the disassembled AP205 VLP protein, while Figure 44 B shows the reassembled particles before purification. Figure 3 C shows an electron micrograph of the purified reassembled AP205 VLPs. The magnification of Figure 3A-C is 200 000 X.

Figure 45 A and B show the reassembled AP205 VLPs analyzed by agarose gel electrophoresis. The samples loaded on the gel from both figures were, from left to right: untreated AP205 VLP, 3 samples with differing amount of AP205 VLP reassembled with CyCpG and purified, and untreated Q $\beta$  VLP. The gel on Figure 45A was stained with ethidium bromide, while the same gel was stained with Coomassie blue in Figure 45 B.

Figure 46 depicts an SDS-PAGE analysis of reassembled AP205 VLP, loaded under non-reducing conditions. 5 samples were loaded on the gel. The samples loaded on the gel are, from left to right: Protein Marker, untreated wt Q $\beta$ , reassembled wt Q $\beta$ , untreated AP205 VLP, reassembled AP205 VLP. The Molecular Weight of the AP205 VLP subunit is 14.0 kDa, while the molecular weight of the Q $\beta$  subunit is 14.3 kDa (both molecular weights calculated with the N-terminal methionine). The disulfide linked multimers are each indicated by an arrow on the figure.

*C. Coupling of p33 epitope (sequence: H2N-KAVYNFATMGGC-COOH, with free N- and C- termini ) to AP205 VLPs reassembled with CyCpG*

Reassembled AP205 VLP obtained as described in part B, and in 20 mM Hepes, 150 mM NaCl, pH 7.4 was reacted at a concentration of 1.4 mg/ml with a 5-fold excess of the crosslinker SMPH diluted from a 50 mM stock in DMSO for 30 minutes at 15 °C. The obtained so-called derivatized AP205 VLP was dialyzed 2 X 2 hours against at least a 1000-fold volume of 20 mM Hepes, 150 mM NaCl, pH 7.4 buffer. The derivatized AP205 was

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reacted at a concentration of 1 mg/ml with either a 2.5-fold, or with a 5-fold excess of peptide, diluted from a 20 mM stock in DMSO, for 2 hours at 15 °C. The sample was subsequently flash frozen in liquid nitrogen for storage.

The result of the coupling reaction is shown in Figure 6. A higher degree of coupling could be achieved by using a 5-fold excess of peptide rather than with a 2.5 fold excess of peptide in the coupling reaction.

Depicted on Figure 47 is the SDS-PAGE analysis of the coupling reaction. The following samples (from left to right) were loaded on the gel: protein marker; derivatized AP205 VLP (d); AP205 VLP coupled with a 2.5-fold excess of peptide, supernatant (s); AP205 VLP coupled with a 2.5-fold excess of peptide, pellet (p); AP205 VLP coupled with a 5-fold excess of peptide, supernatant (s); AP205 VLP coupled with a 5-fold excess of peptide, pellet (p).

### *Example 18*

#### ***Free immunostimulatory nucleic acids but not immunostimulatory nucleic acids packaged in VLPs induce splenomegaly.***

Mice were left untreated or immunized s.c. with 100 µg HBc33 alone, 20 nmol CyCpGpt, 100 µg HBc33 mixed with 20 nmol CyCpGpt, or 100 µg HBc33 packaged with CyCpGpt. Twelve days later, spleens were isolated and spleen weights and splenic cellularity were assessed. CyCpGpt induced a massive increase in spleen weight and number of cells when given alone (Figure 48). No such effect was seen with CyCpGpt packaged in HBc33 although this composition was able to induce protection against viral challenge (see EXAMPLE 4).

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### **EXAMPLE 19**

#### ***In-vivo virus protection assays.***

##### **Vaccinia protection assay**

Groups of three female C57Bl/6 mice were immunized s.c. with 100 µg VLP coupled or fused to p33 alone, mixed with 20 nmol immunostimulatory nucleic acid or packaged with immunostimulatory nucleic acid. To assess antiviral immunity in peripheral tissues, mice were infected 7-9 days later, i.p., with  $1.5 \times 10^6$  pfu recombinant vaccinia virus expressing the LCMV- glycoprotein (inclusive of the p33 peptide). Five days later the ovaries were collected and viral titers determined. Therefore, ovaries were ground with a homogenizer in Minimum Essential Medium (Gibco) containing 5 % fetal bovine serum and supplemented with glutamine, Earls's salts and antibiotics (penicillin/streptomycin/amphotericin). The suspension was titrated in tenfold dilution steps onto BSC40 cells. After overnight incubation at 37°C, the adherent cell layer was stained with a solution consisting of 50% ethanol, 2% crystal violet and 150mM NaCl for visualization of viral plaques. Non-immunized naïve mice were used as control.

##### **LCMV protection assay**

Groups of three female C57Bl/6 mice were immunized s.c. with 100 µg VLP coupled or fused to p33 alone or mixed with adjuvant / 20 nmol CpG oligonucleotide. To examine systemic antiviral immunity mice were infected i.p. 11-13 days later with 200 pfu LCMV-WE. Four days later spleens were isolated and viral titers determined. The spleens were ground with a homogenizer in Minimum Essential Medium (Gibco) containing 2 % fetal bovine serum and supplemented with glutamine, earls's salts and antibiotics (penicillin/streptomycin/amphotericin). The suspension was titrated in tenfold

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dilution steps onto MC57 cells. After incubation for one hour the cells were overlayed with DMEM containing 5% Fetal bovine serum, 1 % methyl cellulose, and antibiotics (penicillin /streptomycin /amphotericin). Following incubation for 2 days at 37°C the cells were assessed for LCMV infection by the intracellular staining procedure (which stains the viral nucleoprotein): Cells were fixed with 4 % Formaldehyde for 30 min followed by a 20 min lysing step with 1% Triton X-100. Incubation for 1 hour with 10 % fetal bovine serum blocked unspecific binding. Cells were stained with a rat anti-LCMV-antibody (VL-4) for 1 hour. A peroxidase-conjugated goat anti-rat-IgG (Jackson ImmunoResearch Laboratories, Inc) was used as secondary antibody followed by a colour reaction with ODP substrate according to standard procedures.

#### *Example 20*

##### ***Different immunostimulatory nucleic acids packaged in VLP fused to antigen result in a potent antigen-specific CTL response and virus protection.***

The fusion protein of HBcAg with the peptide p33 (HBc33) was produced as described in EXAMPLE 1 and packaged with different CpG nucleic acids as described in EXAMPLE 11.

100 µg of vaccines were injected into mice and vaccina titers in the ovaries after recombinant vaccinia challenge were detected as described in EXAMPLE 19. Double stranded CyCpGpt (dsCyCpGpt) was produced by annealing 0.5 mM of DNA oligonucleotides CyCpGpt and CyCpG-rev-pt (Table I) in 15 mM Tris pH7.5 by a 10 min heating step at 80°C and subsequent cooling to RT . Oligonucleotide hybridization was checked on a 20% TBE polyacrylamid gel (Novex).

HBc33 capsids containing CyCpG, NKcPcG, B-CpG and g10gacga-PS did induce CTL responses capable of completely inhibition viral infection (Figure 49, Figure 50). Protection was observed with nucleic acids contained

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phosphodiester or phosphothioate bonds (pt or PS). Even a double stranded oligonucleotide dsCyCpGpt was inducing protection against vaccinia challenge (Figure 49).

### ***Example 21***

#### ***Immunostimulatory nucleic acids packaged in HBcAg and Q $\beta$ VLPs result in a potent antigen-specific CTL response and virus protection.***

The fusion protein of HBcAg with the peptide p33 (HBc33) was produced as described in EXAMPLE 1 and packaged with oligonucleotide B-CpGpt as described in EXAMPLE 11. Peptide p33 was coupled to the RNA phage Q $\beta$  and oligonucleotide B-CpGpt were packaged as described in EXAMPLE 13. 100  $\mu$ g, 30  $\mu$ g, 10  $\mu$ g or 3  $\mu$ g of each vaccine was injected into mice and vaccinia titers in the ovaries after recombinant vaccinia challenge were detected as described in EXAMPLE 19. 100  $\mu$ g and 30  $\mu$ g HBc33 and Qbx33 with packaged B-CpG did induce full protection against viral challenge while at lower concentrations partial or no protection was observed (Figure 51).

### ***Example 22***

#### ***Immunostimulatory nucleic acids packaged in VLPs which are coupled to selfantigens can overcome tolerance to self-antigens.***

Transgenic mice expressing LCMV glycoprotein in pancreatic  $\beta$  islet cells (Ohasi et al., Cell 65, 305-317 (1991)) were immunized with 200 pfu LCMV, 100  $\mu$ g HBc33 mixed with 20 nmol CyCpGpt, 100  $\mu$ g HBc33 packaged with CyCpGpt or 100  $\mu$ g p33 peptide mixed with 20 nmol CyCpGpt as control. Blood glucose levels were measured every four days with the Glucotrend Plus Glucose test kit (Roche). Mice with blood glucose levels larger 12 mM were considered diabetic. Immunization with LCMV induced diabetes in 4/4 animals at day 12. CyCpGpt mixed with HBc33 only caused



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diabetes in 1/3 mice. Two of three mice immunized with HBc33 in which CyCpGpt was packaged develop diabetes at day 12, the third mouse at day 16. Immunization with peptide p33 mixed with CyCpGpt did not induce diabetes in three mice. This clearly shows that immunostimulatory nucleic acid packaged into VLP to which antigens are fused are much more efficient in enhancing a strong CTL response than a mixture of nucleic acid and antigen. They even induced a stronger response than antigen fused to VLP and mixed with the immunostimulatory nucleic acid.

### *Example 23*

***Immunostimulatory nucleic acids packaged in VLPs-coupled to antigens are even more efficient in inducing antigen-specific CD8<sup>+</sup> T cells than VLPs mixed with immunostimulatory nucleic acids.***

C57BL/6 mice were subcutaneously immunized with 100 µg HBc33 alone, mixed with CyCpGpt or, alternatively, packaged with CyCpGpt. Untreated mice served as controls.

8 days after immunization blood lymphocytes were double-stained with PE-labeled p33-tetramers and FITC-coupled monoclonal anti-CD8 antibodies for p33-specific CD8<sup>+</sup> T cell detection and percentage of p33-specific cells on the total CD8<sup>+</sup> T cell population were determined by FACS analysis.

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Table II. Induction of p33-specific CD8<sup>+</sup> T cells after vaccination with p33-VLP mixed or packaged with CpGs. Numbers correspond to means of frequencies (in percent) and standard deviations.

HBc33 with packaged CyCpGpt induced a higher frequency of p33-specific CD8<sup>+</sup> T cells than HBc33 mixed with CyCpGpt (Table II). As the amount of packaged oligonucleotide is much lower (about 1/20) of the amount of oligonucleotide used in the mixed setting this clearly demonstrates that VLPs with packaged immunostimulatory nucleic acids are even more efficient in inducing high numbers of antigen-specific CD8<sup>+</sup> T cells.

#### *Example 24*

***Immunostimulatory nucleic acids packaged in VLPs are even more efficient in inducing CTL responses than VLPs mixed with immunostimulatory nucleic acids.***

Groups of C57BL/6 mice were subcutaneously primed with 100 µg p33-VLP given alone, mixed with 20 nmol CyCpGpt, or, alternatively, packaged with CyCpGpt. For detection of primary ex vivo cytotoxicity, effector cell suspensions were prepared from spleens of vaccinated mice 9 days after priming. EL-4 cells were pulsed with p33 peptide (10<sup>-6</sup> M, 2 h at 37 °C in 2% FCS MEM medium) and used in a 5 h <sup>51</sup>Cr release assay.

Immunization	Frequencies of p33-specific CD8 <sup>+</sup> T cells	Mice per group
Untreated	0.2	2
HBc33	0.3 ± 0.1	4
HBc33 + CyCpGpt (mixed)	2.1 ± 0.9	5
HBc33/CyCpGpt (packaged)	4.3 ± 1.1	5

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Fig 52 shows the primary *ex vivo* cytotoxicity of groups of C57BL/6 mice that were subcutaneously primed with 100 µg p33-VLP given alone (A), mixed with 20 nmol CyCpGpt (B), or, alternatively, packaged with CyCpGpt (C). Nine days later spleen cells were tested for direct *ex vivo* CTL activity in a 5-h <sup>51</sup>Cr-release assay on p33-pulsed (filled symbols) or on unpulsed (open symbols) EL-4 target cells at the indicated effector to target cell ratios. Radioactivity in cell culture supernatants was measured in a Cobra II Counter (Canberra Packard, Downers Grove, IL). Spontaneous release was always < 10 %. Two dilution series of effector cells per mouse were performed. In (A) two mice per group were used, whereas in (B) and (C) data from four mice per group are shown.

Figure 52 clearly demonstrates that 100 µg HBc33 alone did not induce primary *in vivo* CTL response while the same amount HBc33 mixed with 20 nmol CyCpGpt did induce a significant cytotoxicity. However, although the amount of packaged oligonucleotide was much lower (about 1/20) of the amount of oligonucleotide used in the mixed setting cytotoxicity was enhanced when 100 µg HBc33 with packaged CyCpGpt were used for immunization (Figure 52).

### ***Example 25***

#### ***Non-enzymatic hydrolysis of the RNA content of VLPs and packaging of immunostimulatory nucleic acids.***

##### ***ZnSO<sub>4</sub> dependent degradation of the nucleic acid content of a VLP:***

5 mg Qβ VLP (as determined by Bradford analysis) in 20 mM HEPES, pH 7.4, 150 mM NaCl was dialysed either against 2000 ml of 50 mM TrisHCl pH 8.0, 50 mM NaCl, 5% glycerol, 10 mM MgCl<sub>2</sub> or 2000 ml of 4 mM HEPES, pH 7.4, 30 mM NaCl for 2 h at 4°C in SnakeSkin™ pleated dialysis tubing (Pierce, Cat. No. 68035). Each of the dialysis buffers was exchanged once and dialysis was allowed to continue for another 16 h at 4°C. The

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dialysed solution was clarified for 10 minutes at 14 000 rpm (Eppendorf 5417 R, in fixed angle rotor F45-30-11, used in all the following steps) and protein concentration was again determined by Bradford analysis. Q $\beta$  VLPs in 50 mM TrisHCl pH 8.0, 50 mM NaCl, 5% glycerol, 10 mM MgCl<sub>2</sub> were diluted with the corresponding buffer to a final protein concentration of 1 mg/ml whereas Q $\beta$  VLPs in 4 mM HEPES pH 7.4, 30 mM NaCl were diluted with the corresponding buffer to a final protein concentration of 0.5 mg/ml. This capsid-containing solutions were centrifuged again for 10 minutes at 14 000 rpm at 4°C. The supernatants were then incubated with ZnSO<sub>4</sub> which was added to a final concentration of 2.5 mM for 24 h at 60°C in an Eppendorf Thermomixer comfort at 550 rpm. After 24 h the solutions were clarified for 10 minutes at 14000 rpm and the sediment was discarded. The efficiency of the ZnSO<sub>4</sub>-dependent degradation of nucleic acids was confirmed by agarose gelelectrophoresis (Figure 53). The supernatants were dialysed against 5000 ml of 4 mM HEPES pH 7.4, 30 mM NaCl for 2h at 4°C. 5000 ml buffer was exchanged once and dialysis continued over night at 4°C. The dialysed solution was clarified for 10 minutes at 14 000 rpm and 4°C, a negligible sediment was discarded and the protein concentration of the supernatants were determined by Bradford analysis.

Similar results were obtained with copper chloride / phenanthroline / hydrogen peroxide treatment of capsids. Those skilled in the art know alternative non-enzymatic procedures for hydrolysis or RNA.

*Packaging of oligodeoxynucleotides into ZnSO<sub>4</sub>-treated VLPs:*

ZnSO<sub>4</sub>-treated and dialysed Q $\beta$  capsids with a protein concentration (as determined by Bradford analysis) between 0.4 mg/ml and 0.9 mg/ml (which corresponds to a concentration of capsids of 159 nM and 357.5 nM, respectively) were used for the packaging of the oligodeoxynucleotides. The oligodeoxynucleotides were added at a 300-fold molar excess to the of Q $\beta$ -VLP capsids and incubated for 3 h at 37°C in an Eppendorf Thermomixer

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comfort at 550 rpm . After 3 h the reactions were centrifuged for 10 minutes at 14 000 rpm and 4°C. The supernatants were dialysed in Spectra/Por®CE DispoDialyzer with a MWCO 300'000 (Spectrum, Cat. No. 135 526) against 5000 ml of 20 mM HEPES pH 7.4, 150 mM NaCl for 8 h at 4°C. 5000 ml buffer was exchanged once and dialysis continued over night at 4°C. The protein concentration of the dialysed samples were determined by Bradford analysis. Q $\beta$  capsids and their nucleic acid contents were analyzed as described in Examples 11 and 13.

Figure 53 shows the analysis of ZnSO<sub>4</sub>-treated Q $\beta$  VLPs by agarose gelelectrophoresis: Q $\beta$  VLPs which had been purified from *E.coli* and dialysed either against buffer 1 (50 mM TrisHCl pH 8.0, 50 mM NaCl, 5% glycerol, 10 mM MgCl<sub>2</sub>) or buffer 2 (4 mM HEPES, pH 7.4, 30 mM NaCl) were incubated either without or in the presence of 2.5 mM zinc sulfate (ZnSO<sub>4</sub>) for 24 hrs at 60°C. After this treatment equal amounts of the indicated samples (5  $\mu$ g protein) were mixed with loading dye and loaded onto a 0.8% agarose gel. After the run the gel was stained with ethidium bromide. Note that treatment of VLPs with ZnSO<sub>4</sub> causes degradation of the nucleic acid content, while the mock-treated controls were unaffected.

Figure 54 shows the packaging of oligodeoxynucleotides into ZnSO<sub>4</sub>-treated VLPs and analysis of them by agarose gelelectrophoresis. Q $\beta$  VLPs which had been treated with 2.5 mM zinc sulfate ( + ZnSO<sub>4</sub>) were dialysed against 4 mM HEPES, pH 7.4, 30 mM NaCl and incubated for 3 hrs at 37°C with an excess of oligodeoxynucleotides (due to the dialysis the concentration of ZnSO<sub>4</sub> was decreased by an order of 10<sup>6</sup>, therefore its indicated only in parenthesis) After this incubation in presence of oligodeoxynucleotides, equal amounts of the indicated samples (5  $\mu$ g protein) were mixed with loading dye and loaded onto a 0.8% agarose gel. After the run the gel was stained with ethidium bromide. Note that adding of oligodeoxynucleotides to ZnSO<sub>4</sub>-treated Q $\beta$  VLPs could restore the electrophoretical behaviour of the so treated capsids when compared to untreated Q $\beta$  capsids which had been purified from *E.coli*.

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Figure 55 shows the analysis of nucleic acid content of ZnSO<sub>4</sub>- and oligodeoxynucleotide treated Q $\beta$  VLPs by Benzonase and proteinase K digestion and polyacrylamide TBE/Urea gelelectrophoresis: Oligodeoxynucleotides were packaged into ZnSO<sub>4</sub>-treated Q $\beta$  VLPs as described above. 25  $\mu$ g of these VLPs were digested with 25  $\mu$ l Benzonase (Merck, Cat. No. 1.01694.0001) according to the manufactures instructions. After heat-inactivation of the nuclease (30 minutes at 80°C) the VLPs were treated with Proteinase K (final enzyme concentration was 0.5 mg/ml) according to the manufactures instructions. After 3 hrs the equivalent of 2  $\mu$ g Q $\beta$  VLPs which had been digested by Benzonase and proteinase K were mixed with TBE-Urea sample buffer and loaded on a 15% polyacrylamide TBE-Urea gel (Novex<sup>®</sup>, Invitrogen Cat. No. EC6885). The capsids loaded in lane 2 were treated with 2.5 mM ZnSO<sub>4</sub> in presence of buffer 1 (see above), while the capsids loaded in lane 3 were treated with 2.5 mM ZnSO<sub>4</sub> in presence of buffer 2 (see above). As qualitative as well as quantitative standard, 1 pmol, 5 pmol and 10 pmol of the oligodeoxynucleotide which was used for the reassembly reaction, was loaded onto the same gel (lanes 4 – 6). As control, Q $\beta$  capsids which had been purified from *E.coli* were treated exactly the same and analyzed on the same polyacrylamide TBE-Urea gel (lane 1) . After the run was completed, the gel was fixed, equilibrated to neutral pH and stained with SYBR-Gold (Molecular Probes Cat. No. S-11494). Note that intact Q $\beta$  VLPs (which had been purified from *E.coli*) did not contain nucleic acids of similar size than those which had been extracted from ZnSO<sub>4</sub>- and oligodeoxynucleotide treated Q $\beta$  capsids. In addition, nucleic acids isolated from the latter VLPs were comigrating with the oligodeoxynucleotides which had been used in the reassembly reaction. This results confirmed that the used oligodeoxynucleotides were packaged into ZnSO<sub>4</sub>-treated Q $\beta$  capsids.

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***Example 26***

***VLPs containing immunostimulatory nucleic acids induce T cell responses that can be boosted by viral vectors: LCMV.***

Mice were subcutaneously primed with 20 µg p33-VLPs containing immunostimulatory nucleic acids. Before immunization, p33-VLP preparations were extensively purified from unbound CpG-oligonucleotides via dialysis (see Example 2 and Figure 5). 12 days later, blood was taken and frequencies of p33-specific T cells were determined by tetramer staining. The mice were boosted with 200 pfu of live LCMV strain WE and frequencies of specific T cells were determined 5 days later. Frequencies before boost were 3.5% +/- 1.8% and after boost 15.5% +/- 1.9%.

***Example 27***

***VLPs containing immunostimulatory nucleic acids induce T cell responses that can be boosted by viral vectors: recombinant vaccinia virus***

Mice are subcutaneously primed with 20 µg p33-VLPs containing immunostimulatory nucleic acids. Before immunization, p33-VLP preparations are extensively purified from unbound CpG-oligonucleotides via dialysis (see Example 2 and Figure 5). 12 days later, blood is taken and frequencies of p33-specific T cells are determined by tetramer staining. The mice are boosted with 10<sup>6</sup> pfu of recombinant vaccinia virus expressing LCMV-GP and frequencies of specific T cells are determined 5 days later.

***Example 28***

***VLPs containing immunostimulatory nucleic acids induce T cell responses that can be boosted by viral vectors: recombinant canary pox virus.***

Mice are subcutaneously primed with 20 µg p33-VLPs containing immunostimulatory nucleic acids. Before immunization, p33-VLP

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preparations are extensively purified from unbound CpG-oligonucleotides via dialysis (see Example 2 and Figure 5). 12 days later, blood is taken and frequencies of p33-specific T cells are determined by tetramer staining. The mice are boosted with  $10^7$  pfu of recombinant canary pox virus expressing LCMV-GP and frequencies of specific T cells are determined 5 days later.

#### ***Example 29***

##### ***VLPs containing immunostimulatory nucleic acids can boost T cell responses.***

Mice are infected intravenously with recombinant vaccinia virus expressing LCMV-GP. 20 days later, blood is taken and frequencies of p33-specific T cells are determined by tetramer staining. The mice are boosted the same day with p33-VLP preparations containing immunostimulatory nucleic acids (see Example 2 and Figure 5) and frequencies of specific T cells are determined 5 days later.

#### ***Example 30***

##### ***Packaging of immunostimulatory ribonucleic acids into VLPs.***

Immunostimulatory ribonucleic acids such as poly (I:C) (Sigma) or synthetic double-stranded 30 mer of polyinosinic acid and polycytidylic acid either with phosphodiester or phosphorothiate backbone are dissolved in water. Alternatively, polydeoxyinosinic acid and polydeoxyinosinic acid are used to prepare a double stranded poly(I:C) analogon. HBc33 VLPs and Q $\beta$  VLPs are treated with RNase as described in Examples 11, 13 or 25 and nucleic acids are added at 1, 10 and 100 nmol/ml in 0.2x HBS and incubated for 3 h at 37°C in a thermomixer. Excess nucleic acids are removed by enzymatic hydrolysis or dialysis and analysed as described in Example 11, 13 and 25.



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Alternatively, immunostimulatory ribonucleic acids and their analogs are packaged during reassembly of Q $\beta$  coat proteins as described in Examples 14, 15, 16. Reassembly is performed by adding  $\beta$ -mercaptoethanol to the 10 ml dimer fraction to a final concentration of 10%, and 300  $\mu$ l of a solution of nucleic acid, resulting in a 1, 10 and 100 molar excess over capsid concentration, are added. The reassembly mixtures are first dialyzed against 30 ml NET buffer containing 10% beta-mercaptoethanol for 2 hours at 4°C, and then dialyzed in a continuous mode, with a flow of NET buffer of 8 ml/h over 4 days at 4°C. The reassembly mixtures are then desalted against water by dialysis, with 6 buffer exchanges (4 X 100 ml, 2 X 1 liter). Reassembled Q $\beta$  VLPs are then isolated by sucrose gradient centrifugation as described in Example 14 or by gel filtration as described in Example 16.

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***What Is Claimed Is:***

1. A composition for enhancing an immune response in an animal comprising:

- (a) a virus-like particle; and
- (b) an immunostimulatory substance;

wherein said immunostimulatory substance is bound to said virus-like particle.

2. The composition of claim 1 further comprising at least one antigen, wherein said antigen is bound to said virus-like particle.

3. The composition of claim 1, wherein said immunostimulatory substance is a toll-like receptor activating substance.

4. The composition of claim 1, wherein said immunostimulatory substance is a cytokine secretion inducing substance.

5. The composition of claim 3, wherein said toll-like receptor activating substance is selected from the group consisting of, or alternatively consists essentially of:

- (a) immunostimulatory nucleic acids;
- (b) peptidoglycans;
- (c) lipopolysaccharides;
- (d) lipoteichonic acids;
- (e) imidazoquinoline compounds;
- (f) flagellines;
- (g) lipoproteins;
- (h) immunostimulatory organic molecules;
- (i) unmethylated CpG-containing oligonucleotides; and
- (j) any mixtures of at least one substance of (a), (b), (c), (d), (e), (f), (g), (h) and/or (i).

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6. The composition of claim 5, wherein said immunostimulatory nucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

7. The composition of claim 6, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

8. The composition of claim 6, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

9. The composition of claim 1, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

10. The composition of claim 1, wherein said virus-like particle lacks a lipoprotein-containing envelope.

11. The composition of claim 1, wherein said virus-like particle is a recombinant virus-like particle.

12. The composition of claim 11, wherein said virus-like particle is selected from the group consisting of:

- (a) recombinant proteins of Hepatitis B virus;

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- (b) recombinant proteins of measles virus;
- (c) recombinant proteins of Sinbis virus;
- (d) recombinant proteins of Rotavirus;
- (e) recombinant proteins of Foot-and-Mouth-Disease virus;
- (f) recombinant proteins of Retrovirus;
- (g) recombinant proteins of Norwalk virus;
- (h) recombinant proteins of human Papilloma virus;
- (i) recombinant proteins of BK virus;
- (j) recombinant proteins of bacteriophages;
- (k) recombinant proteins of RNA-phages;
- (l) recombinant proteins of Q $\beta$ -phage;
- (m) recombinant proteins of GA-phage
- (n) recombinant proteins of fr-phage;
- (o) recombinant proteins of AP 205-phage;
- (p) recombinant proteins of Ty; and
- (q) fragments of any of the recombinant proteins from (a) to (p).

13. The composition of claim 12, wherein said virus-like particle is the Hepatitis B virus core protein.

14. The composition of claim 12, wherein said virus-like particle is the BK virus VP1 protein.

15. The composition of claim 1, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage.

16. The composition of claim 15, wherein said RNA-phage is selected from the group consisting of:

- (a) bacteriophage Q $\beta$ ;
- (b) bacteriophage R17;

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- (c) bacteriophage fr;
- (d) bacteriophage GA;
- (e) bacteriophage SP;
- (f) bacteriophage MS2;
- (g) bacteriophage M11;
- (h) bacteriophage MX1;
- (i) bacteriophage NL95;
- (k) bacteriophage f2;
- (l) bacteriophage PP7; and
- (m) bacteriophage AP205.

17. The composition of claim 1, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage Q $\beta$ .

18. The composition of claim 1, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage AP 205.

19. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide comprises the sequence:



wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> are any nucleotide.

20. The composition of claim 19, wherein at least one of said nucleotide X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> has a phosphate backbone modification.

21. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide comprises, or alternatively consists essentially of, or alternatively consists of the sequence selected from the group consisting of:

- (a) TCCATGACGTTCTGAATAAT;
- (b) TCCATGACGTTCTGACGTT;
- (c) GGGGTCAACGTTGAGGGGG;

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- (d) GGGGGGGGGGGACGATCGTCGGGGGGGGGG;  
and
- (e) “dsCyCpG-253” as described in Table 1.

22. The composition of claim 21, wherein said unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

23. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide is palindromic.

24. The composition of claim 23, wherein said palindromic unmethylated CpG-containing oligonucleotide comprises, or alternatively consists essentially of, or alternatively consists of the sequence GGGGTCAACGTTGAGGGGG.

25. The composition of claim 24, wherein said palindromic unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

26. The composition of claim 1, wherein said immunostimulatory substance is non-covalently bound to said virus-like particle.

27. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide is non-covalently bound to said virus-like particle.

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28. The composition of claim 5, wherein said immunostimulatory nucleic acid is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and a RNA binding site.

29. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and a RNA binding site.

30. The composition of claim 29, wherein said oligonucleotide binding site is a non-naturally occurring oligonucleotide binding site.

31. The composition of claim 29, wherein said virus-like particle site comprises an arginine-rich repeat.

32. The composition of claim 5, wherein said immunostimulatory nucleic acid contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

33. The composition of claim 9, wherein said unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

34. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 6 to about 100,000 nucleotides.

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35. The composition of claim 34, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 6 to about 2000 nucleotides.

36. The composition of claim 35, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 20 to about 2000 nucleotides.

37. The composition of claim 36, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 20 to about 300 nucleotides.

38. The composition of claim 37, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises 20 to 100 nucleotides.

39. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 2000 nucleotides.

40. The composition of claim 39, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 1000 nucleotides.

41. The composition of claim 40, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 500 nucleotides.

42. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a recombinant oligonucleotide.



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43. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a genomic oligonucleotide.

44. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a synthetic oligonucleotide.

45. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a plasmid-derived oligonucleotide.

46. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a single-stranded oligonucleotide.

47. The composition of claim 5, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a double-stranded oligonucleotide.

48. The composition of claim 2, wherein said at least one antigen or antigenic determinant is bound to said virus-like particle by at least one covalent bond.

49. The composition of claim 2, wherein said at least one antigen or antigenic determinant is bound to said virus-like particle by at least one covalent bond, and wherein said covalent bond is a non-peptide bond.

50. The composition of claim 2, wherein said at least one antigen or antigenic determinant is fused to said virus-like particle.

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51. The composition of claim 2, wherein said antigen or antigenic determinant further comprises at least one second attachment site being selected from the group consisting of:

- (a) an attachment site not naturally occurring with said antigen or antigenic determinant; and
- (b) an attachment site naturally occurring with said antigen or antigenic determinant.

52. The composition of claim 2 further comprising an amino acid linker, wherein said amino acid linker comprises, or alternatively consists of, said second attachment site.

53. The composition of claim 2, wherein said antigen is selected from the group consisting of:

- (a) polypeptides;
- (b) carbohydrates;
- (c) steroid hormones; and
- (d) organic molecules.

54. The composition of claim 53, wherein said antigen is an organic molecule.

55. The composition of claim 54, wherein said organic molecule is selected from the group consisting of:

- (a) codeine;
- (b) fentanyl;
- (c) heroin;
- (d) morphium;
- (e) amphetamine;
- (f) cocaine;

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- (g) methylenedioxymethamphetamine;
- (h) methamphetamine;
- (i) methylphenidate;
- (j) nicotine;
- (k) LSD;
- (l) mescaline;
- (m) psilocybin; and
- (n) tetrahydrocannabinol.

56. The composition of claim 2, wherein said antigen is a recombinant antigen.

57. The composition of claim 2, wherein said antigen is derived from the group consisting of:

- (a) viruses;
- (b) bacteria;
- (c) parasites;
- (d) prions;
- (e) tumors;
- (f) self-molecules;
- (g) non-peptidic hapten molecules
- (h) allergens; and
- (i) hormones.

58. The composition of claim 57, wherein said antigen is a tumor antigen.

59. The composition of claim 58, wherein said tumor antigen is selected from the group consisting of:

- (a) Her2;
- (b) GD2;

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- (c) EGF-R;
- (d) CEA;
- (e) CD52;
- (f) CD21;
- (g) human melanoma protein gp100;
- (h) human melanoma protein melan-A/MART-1;
- (i) tyrosinase;
- (j) NA17-A nt protein;
- (k) MAGE-3 protein;
- (l) p53 protein;
- (m) HPV16 E7 protein; and
- (n) antigenic fragments of any of the tumor antigens from (a) to (m).

60. The composition of claim 2, wherein said antigen is bound to said virus-like particle by way of a linking sequence.

61. The composition of claim 60, wherein said virus-like particle is the Hepatitis B virus core protein.

62. The composition of claim 60, wherein said virus-like particle is the BK virus core protein.

63. The composition of claim 2, wherein said antigen is a cytotoxic T cell epitope, a Th cell epitope or a combination of at least two of said epitopes, wherein said at least two epitopes are bound directly or by way of a linking sequence.

64. The composition of claim 63, wherein said cytotoxic T cell epitope is a viral or a tumor cytotoxic T cell epitope.

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65. The composition of claim 63, wherein said antigen is bound to said virus-like particle by way of a linking sequence.

66. The composition of claim 63, wherein said virus-like particle is the Hepatitis B virus core protein.

67. The composition of claim 66, wherein said cytotoxic T cell epitope is fused to the C-terminus of said Hepatitis B virus core protein.

68. The composition of claim 67, wherein said cytotoxic T cell epitope is fused to the C-terminus of said Hepatitis B virus core protein by way of a linking sequence.

69. The composition of claim 63, wherein said virus-like particle is the BK virus VP1 protein.

70. The composition of claim 69, wherein said cytotoxic T cell epitope is fused to the C-terminus of said BK virus VP1 protein.

71. The composition of claim 70, wherein said cytotoxic T cell epitope is fused to the C-terminus of said BK virus VP1 protein by way of a linking sequence.

72. A method for enhancing an immune response in an animal comprising introducing into said animal a composition comprising:

- (a) a virus-like particle; and
- (b) an immunostimulatory substance;

wherein said immunostimulatory substance is bound to said virus-like particle.

73. The method of claim 72, wherein said composition further comprises an antigen, wherein said antigen is bound to said virus-like particle.

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74. The method of claim 72, wherein said immunostimulatory substance is a toll-like receptor activating substance.

75. The method of claim 72, wherein said immunostimulatory substance is a cytokine secretion inducing substance.

76. The method of claim 74, wherein said toll-like receptor activating substance is selected from the group consisting of, or alternatively consists essentially of:

- (a) immunostimulatory nucleic acids;
- (b) peptidoglycans;
- (c) lipopolysaccharides;
- (d) lipoteichonic acids;
- (e) imidazoquinoline compounds;
- (f) flagellines;
- (g) lipoproteins;
- (h) immunostimulatory organic molecules;
- (i) unmethylated CpG-containing oligonucleotides; and
- (j) any mixtures of at least one substance of (a), (b), (c), (d), (e), (f), (g), (h) and/or (i).

77. The method of claim 76, wherein said immunostimulatory nucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

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78. The method of claim 77, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

79. The method of claim 77, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

80. The method of claim 72, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

81. The method of claim 72, wherein said virus-like particle lacks a lipoprotein-containing envelope.

82. The method of claim 72, wherein said virus-like particle is a recombinant virus-like particle.

83. The method of claim 82, wherein said virus-like particle is selected from the group consisting of:

- (a) recombinant proteins of Hepatitis B virus;
- (b) recombinant proteins of measles virus;
- (c) recombinant proteins of Sinbis virus;
- (d) recombinant proteins of Rotavirus;
- (e) recombinant proteins of Foot-and-Mouth-Disease virus;
- (f) recombinant proteins of Retrovirus;
- (g) recombinant proteins of Norwalk virus;
- (h) recombinant proteins of human Papilloma virus;
- (i) recombinant proteins of BK virus;
- (j) recombinant proteins of bacteriophages;
- (k) recombinant proteins of RNA-phages;
- (l) recombinant proteins of Q $\beta$ -phage;

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- (m) recombinant proteins of GA-phage;
- (n) recombinant proteins of fr-phage;
- (o) recombinant proteins of AP 205-phage;
- (p) recombinant proteins of Ty; and
- (q) fragments of any of the recombinant proteins from (a) to (p).

84. The method of claim 83, wherein said virus-like particle is the Hepatitis B virus core protein.

85. The method of claim 83, wherein said virus-like particle is the BK virus VP1 protein.

86. The method of claim 72, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage.

87. The method of claim 86, wherein said RNA-phage is selected from the group consisting of:

- (a) bacteriophage Q $\beta$ ;
- (b) bacteriophage R17;
- (c) bacteriophage fr;
- (d) bacteriophage GA;
- (e) bacteriophage SP;
- (f) bacteriophage MS2;
- (g) bacteriophage M11;
- (h) bacteriophage MX1;
- (i) bacteriophage NL95;
- (k) bacteriophage f2;
- (l) bacteriophage PP7; and
- (m) bacteriophage AP205.



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88. The method of claim 72, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage Q $\beta$ .

89. The method of claim 72, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage AP 205.

90. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide comprises the sequence:



wherein  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  are any nucleotide.

91. The method of claim 90, wherein at least one of said nucleotide  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  has a phosphate backbone modification.

92. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide comprises, or alternatively consists essentially of, or alternatively consists of the sequence selected from the group consisting of:

- (a) TCCATGACGTTTCCTGAATAAT;
- (b) TCCATGACGTTTCCTGACGTT;
- (c) GGGGTCAACGTTGAGGGGG;
- (d) GGGGGGGGGGACGATCGTCGGGGGGGGG;
- and
- (e) "dsCyCpG-253" as described in Table 1.

93. The method of claim 92, wherein said unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

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94. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide is palindromic.

95. The method of claim 94, wherein said palindromic unmethylated CpG-containing oligonucleotide comprises, or alternatively consists essentially of, or alternatively consists of the sequence GGGGTCAACGTTGAGGGGG.

96. The method of claim 95, wherein said palindromic unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

97. The method of claim 72, wherein said immunostimulatory substance is non-covalently bound to said virus-like particle.

98. The method of claim 72, wherein said virus-like particle is produced in a bacterial expression system.

99. The method of claim 72, wherein said virus-like particle is produced in a yeast expression system.

100. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide is non-covalently bound to said virus-like particle.

101. The method of claim 72, wherein said immunostimulatory substance is packaged, preferably enclosed by said virus-like particle.

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102. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide is packaged, preferably enclosed by said virus-like particle.

103. The method of claim 76, wherein said immunostimulatory nucleic acid is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and a RNA binding site

104. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and a RNA binding site.

105. The method of claim 104, wherein said oligonucleotide binding site is a non-naturally occurring oligonucleotide binding site.

106. The method of claim 104, wherein said virus-like particle site comprises an arginine-rich repeat.

107. The method of claim 76, wherein said immunostimulatory nucleic acid contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

108. The method of claim 80, wherein said unmethylated CpG-containing oligonucleotide contains one or more phosphorothioate modifications of the phosphate backbone or wherein each phosphate moiety of said phosphate backbone of said oligonucleotide is a phosphorothioate modification.

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109. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 6 to about 100,000 nucleotides.

110. The method of claim 109, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 6 to about 2000 nucleotides.

111. The method of claim 110, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 20 to about 2000 nucleotides.

112. The method of claim 111, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises about 20 to about 300 nucleotides.

113. The method of claim 112, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises 20 to 100 nucleotides.

114. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 2000 nucleotides.

115. The method of claim 114, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 1000 nucleotides.

116. The method of claim 115, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide comprises more than 100 to about 500 nucleotides.

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117. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a recombinant oligonucleotide.

118. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a genomic oligonucleotide.

119. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a synthetic oligonucleotide.

120. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a plasmid-derived oligonucleotide.

121. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a single-stranded oligonucleotide.

122. The method of claim 76, wherein said immunostimulatory nucleic acid, and preferably said unmethylated CpG-containing oligonucleotide is a double-stranded oligonucleotide.

123. The method of claim 73, wherein said at least one antigen or antigenic determinant is bound to said virus-like particle by at least one covalent bond.

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124. The method of claim 73, wherein said at least one antigen or antigenic determinant is bound to said virus-like particle by at least one covalent bond, and wherein said covalent bond is a non-peptide bond.

125. The method of claim 73, wherein said at least one antigen or antigenic determinant is fused to said virus-like particle.

126. The method of claim 73, wherein said antigen or antigenic determinant further comprises at least one second attachment site selected from the group consisting of:

- (a) an attachment site not naturally occurring with said antigen or antigenic determinant; and
- (b) an attachment site naturally occurring with said antigen or antigenic determinant.

127. The method of claim 73, further comprising an amino acid linker, wherein said amino acid linker comprises, or alternatively consists of, said second attachment site.

128. The method of claim 73, wherein said antigen is selected from the group consisting of:

- (a) polypeptides;
- (b) carbohydrates;
- (c) steroid hormones; and
- (d) organic molecules.

129. The method of claim 128, wherein said antigen is an organic molecule.

130. The method of claim 129, wherein said organic molecule is selected from the group consisting of:

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- (a) codeine;
- (b) fentanyl;
- (c) heroin;
- (d) morphium;
- (e) amphetamine;
- (f) cocaine;
- (g) methylenedioxymethamphetamine;
- (h) methamphetamine;
- (i) methylphenidate;
- (j) nicotine;
- (k) LSD;
- (l) mescaline;
- (m) psilocybin; and
- (n) tetrahydrocannabinol.

131. The method of claim 73, wherein said antigen is a recombinant antigen.

132. The method of claim 73, wherein said antigen is derived from the group consisting of:

- (a) viruses;
- (b) bacteria;
- (c) parasites;
- (d) prions;
- (e) tumors;
- (f) self-molecules;
- (g) non-peptidic hapten molecules
- (h) allergens; and
- (i) hormones.

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133. The method of claim 132, wherein said antigen is a tumor antigen.

134. The method of claim 133, wherein said tumor antigen is selected from the group consisting of:

- (a) Her2;
- (b) GD2;
- (c) EGF-R;
- (d) CEA;
- (e) CD52;
- (f) CD21;
- (g) human melanoma protein gp100;
- (h) human melanoma protein melan-A/MART-1;
- (i) tyrosinase;
- (j) NA17-A nt protein;
- (k) MAGE-3 protein;
- (l) p53 protein;
- (m) HPV16 E7 protein; and
- (m) antigenic fragments of any of the tumor antigens from (a) to (m).

135. The method of claim 73, wherein said antigen is bound to said virus-like particle by way of a linking sequence.

136. The method of claim 135, wherein said virus-like particle is the Hepatitis B virus core protein.

137. The method of claim 135, wherein said virus-like particle is the BK virus VP1 protein.



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138. The method of claim 73, wherein said antigen is a cytotoxic T cell epitope, a Th cell epitope or a combination of at least two of said epitopes, wherein said at least two epitopes are linked directly or by way of a linking sequence.

139. The method of claim 138, wherein said cytotoxic T cell epitope is a viral or a tumor cytotoxic T cell epitope.

140. The method of claim 138, wherein said antigen is bound to said virus-like particle by way of a linking sequence.

141. The method of claim 138, wherein said virus-like particle is the Hepatitis B virus core protein.

142. The method of claim 141, wherein said cytotoxic T cell epitope is fused to the C-terminus of said Hepatitis B virus core protein.

143. The method of claim 142, wherein said cytotoxic T cell epitope is fused to the C-terminus of said Hepatitis B virus core protein by way of a linking sequence.

144. The method of claim 138, wherein said virus-like particle is a BK virus VP1 protein.

145. The method of claim 144, wherein said cytotoxic T cell epitope is fused to the C-terminus of said BK virus VP1 protein.

146. The method of claim 145, wherein said cytotoxic T cell epitope is fused to the C-terminus of said BK virus VP1 protein by way of a linking sequence.

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147. The method of claim 72, wherein said immune response is an enhanced B cell response.

148. The method of claim 72, wherein said immune response is an enhanced T cell response.

149. The method of claim 148, wherein said T cell response is a CTL response.

150. The method of claim 148, wherein said T cell response is a Th cell response.

151. The method of claim 150, wherein said Th cell response is a Th1 cell response.

152. The method of claim 72, wherein said animal is a mammal.

153. The method of claim 152, wherein said mammal is a human.

154. The method of claim 72, wherein said composition is introduced into said animal subcutaneously, intramuscularly, intravenously, intranasally or directly into the lymph node.

155. A method of producing a composition for enhancing an immune response in an animal comprising a virus-like particle and an immunostimulatory substance bound to said virus-like particle which comprises:

- (a) incubating said virus-like particle with said immunostimulatory substance;
- (b) adding RNase; and
- (c) purifying said composition.

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156. The method of claim 155, wherein said immunostimulatory substance is an immunostimulatory nucleic acid selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

157. The method of claim 156, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

158. The method of claim 156, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

159. The method of claim 155, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

160. The method of claim 155, wherein said virus-like particle is produced in a bacterial expression system.

161. The method of claim 155, wherein said RNase is RNase A.

162. A method of producing a composition for enhancing an immune response in an animal comprising a virus-like particle and an immunostimulatory substance bound to said virus-like particle which comprises:

- (a) incubating said virus-like particle with RNase;

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- (b) adding said immunostimulatory substance; and
- (c) purifying said composition.

163. The method of claim 162, wherein said immunostimulatory substance is an immunostimulatory nucleic acid selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

164. The method of claim 163, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

165. The method of claim 163, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

166. The method of claim 162, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

167. The method of claim 162, wherein said virus-like particle is produced in a bacterial expression system.

168. The method of claim 162, wherein said RNase is RNase A.

169. A method of producing a composition for enhancing an immune response in an animal comprising a virus-like particle and an

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immunostimulatory substance bound to said virus-like particle which comprises:

- (a) disassembling said virus-like particle;
- (b) adding said immunostimulatory substance; and
- (c) reassembling said virus-like particle.

170. The method of claim 169, wherein said immunostimulatory substance is an immunostimulatory nucleic acid selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

171. The method of claim 170, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

172. The method of claim 170, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

173. The method of claim 169, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

174. The method of claim 169 further comprising removing nucleic acids of said disassembled virus-like particle.

175. The method of claim 169 further comprising purifying said composition after reassembly (c).

- 183 -

176. A method of producing a composition for enhancing an immune response in an animal comprising a virus-like particle and an immunostimulatory substance bound to said virus-like particle which comprises:

- (a) incubating said virus-like particle with solutions comprising metal ions capable of hydrolyzing the nucleic acids of said virus-like particle;
- (b) adding said immunostimulatory substance; and
- (c) purifying said composition.

177. The method of claim 176, wherein said immunostimulatory substance is an immunostimulatory nucleic acid selected from the group consisting of, or alternatively consists essentially of:

- (a) ribonucleic acids;
- (b) deoxyribonucleic acids;
- (c) chimeric nucleic acids; and
- (d) any mixtures of at least one nucleic acid of (a), (b) and/or (c).

178. The method of claim 177, wherein said ribonucleic acid is poly-(I:C) or a derivative thereof.

179. The method of claim 177, wherein said deoxyribonucleic acid is selected from the group consisting of, or alternatively consists essentially of:

- (a) unmethylated CpG-containing oligonucleotides; and
- (b) oligonucleotides free of unmethylated CpG motifs.

180. The method of claim 176, wherein said immunostimulatory substance is an unmethylated CpG-containing oligonucleotide.

- 184 -

181. The method of claim 176, wherein said metal ions are selected from the group consisting of:

- (a) zinc (Zn) ions;
- (b) copper (Cu) ions;
- (c) iron (Fe) ions; and
- (d) any mixtures of at least one ion of (a), (b) and/or (c).

182. A vaccine comprising an immunologically effective amount of the composition of claim 1 together with a pharmaceutically acceptable diluent, carrier or excipient.

183. The vaccine of claim 182 further comprising an adjuvant.

184. A method of immunizing or treating an animal comprising administering to said animal an immunologically effective amount of the vaccine of claim 182.

185. The method of claim 184, wherein said animal is a mammal.

186. The method of claim 185, wherein said mammal is a human.

187. A vaccine comprising an immunologically effective amount of the composition of claim 2 together with a pharmaceutically acceptable diluent, carrier or excipient.

188. The vaccine of claim 187 further comprising an adjuvant.

189. A method of immunizing or treating an animal comprising administering to said animal an immunologically effective amount of the vaccine of claim 187.

- 185 -

190. The method of claim 189, wherein said animal is a mammal.

191. The method of claim 190, wherein said mammal is a human.

192. A method of immunizing or treating an animal comprising priming a T cell response in said animal by administering an immunologically effective amount of the vaccine of claim 182.

193. The method of claim 192, further comprising the step of boosting the immune response in said animal.

194. The method of claim 193, wherein said boosting is effected by administering an immunologically effective amount of a vaccine of claim 182 or an immunologically effective amount of a heterologous vaccine.

195. The method of claim 194, wherein said heterologous vaccine is a DNA vaccine.

196. A method of immunizing or treating an animal comprising boosting a T cell response in said animal by administering an immunologically effective amount of the vaccine of claim 182.

197. The method of claim 196, further comprising the step of priming a T cell response in said animal.

198. The method of claim 197, wherein said priming is effected by administering an immunologically effective amount of a vaccine of claim 182 or an immunologically effective amount of a heterologous vaccine.

199. The method of claim 198, wherein said heterologous vaccine is a DNA vaccine.



- 186 -

200. A method of immunizing or treating an animal comprising priming a T cell response in said animal by administering an immunologically effective amount of the vaccine of claim 187.

201. The method of claim 200 further comprising the step of boosting the immune response in said animal.

202. The method of claim 201, wherein said boosting is effected by administering an immunologically effective amount of a vaccine of claim 187 or an immunologically effective amount of a heterologous vaccine.

203. The method of claim 202, wherein said heterologous vaccine is a DNA vaccine or a viral vaccine or a canary pox vaccine.

204. A method of immunizing or treating an animal comprising boosting a T cell response in said animal by administering an immunologically effective amount of the vaccine of claim 187.

205. The method of claim 204, further comprising the step of priming a T cell response in said animal.

206. The method of claim 205, wherein said priming is effected by administering an immunologically effective amount of a vaccine of claim 187 or an immunologically effective amount of a heterologous vaccine.

207. The method of claim 206, wherein said heterologous vaccine is a DNA vaccine or a viral vaccine or a canary pox vaccine.

Fig. 1

A

5' TCCATGACGTTCCCTGAATAAT 3'

B

1 atggacattg acccttataa agaatttggg gctactgtgg agttactctc gtttttgcc tctgacttct ttcctccgt cagagatctc ctgacacccg  
M D I D P Y K E F G A T V E L L S F L P S D F F P S V R D L L D T

101 cctcagctct gtatcgagaa gccttagagt ctccctgagca ttgctcacct caccatactg cactcaggca agccattctc tgctgggggg aattgatgac  
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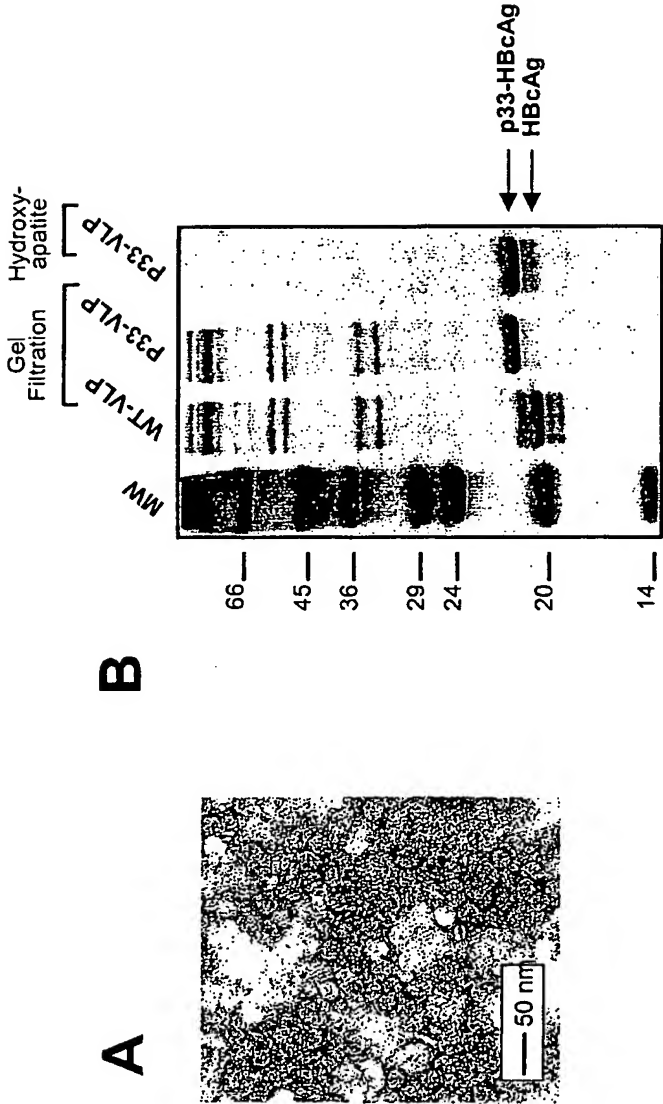
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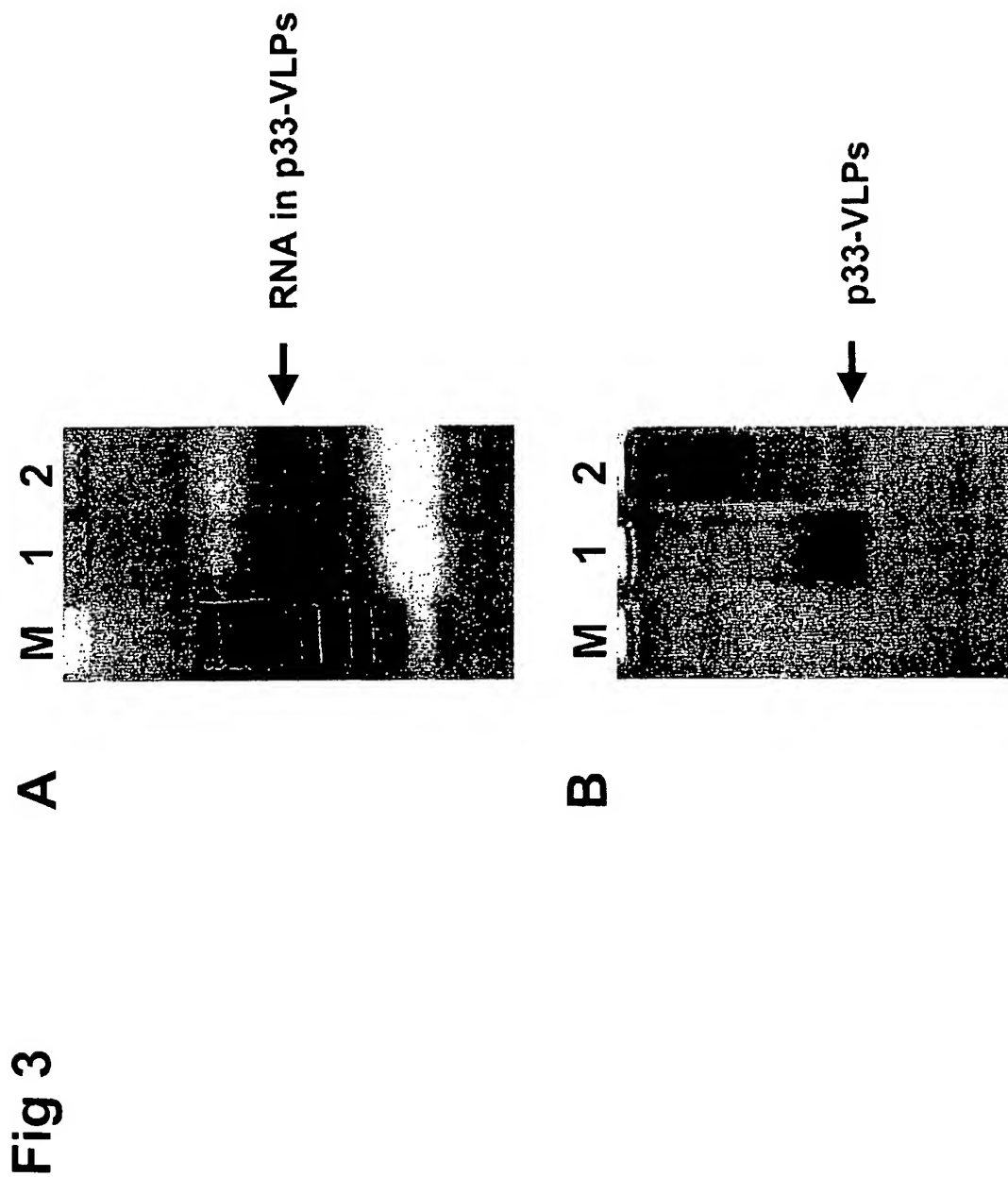
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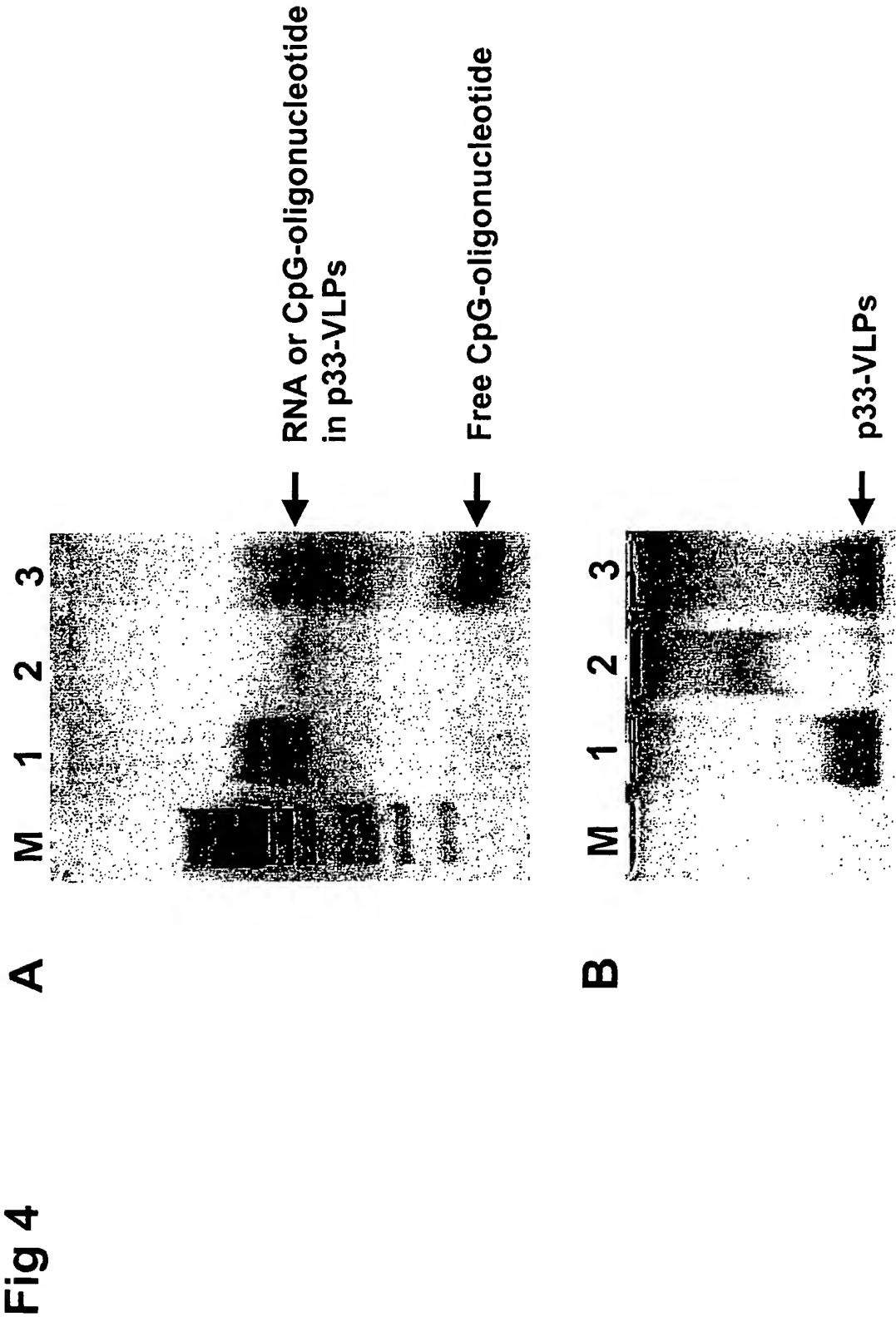
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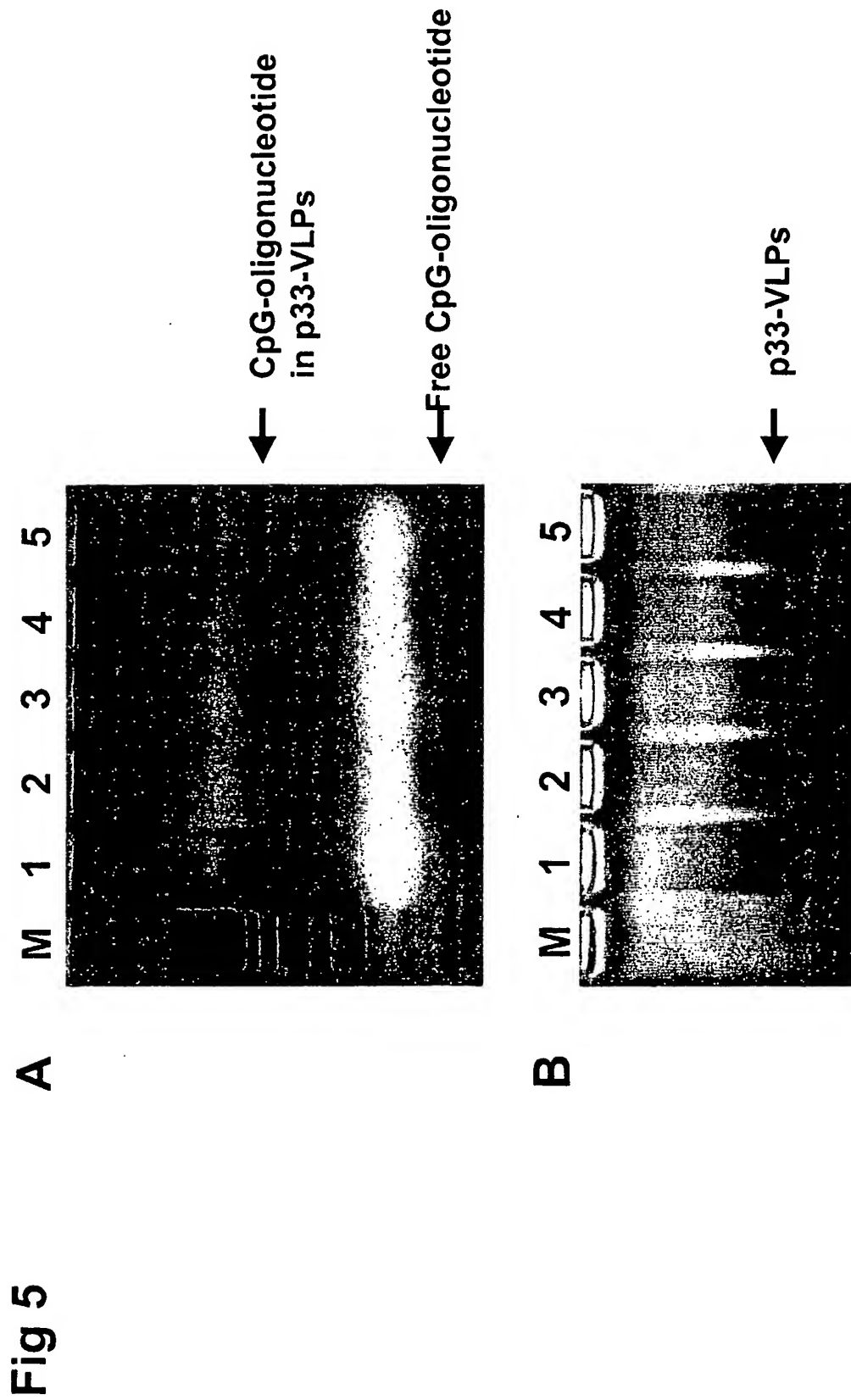
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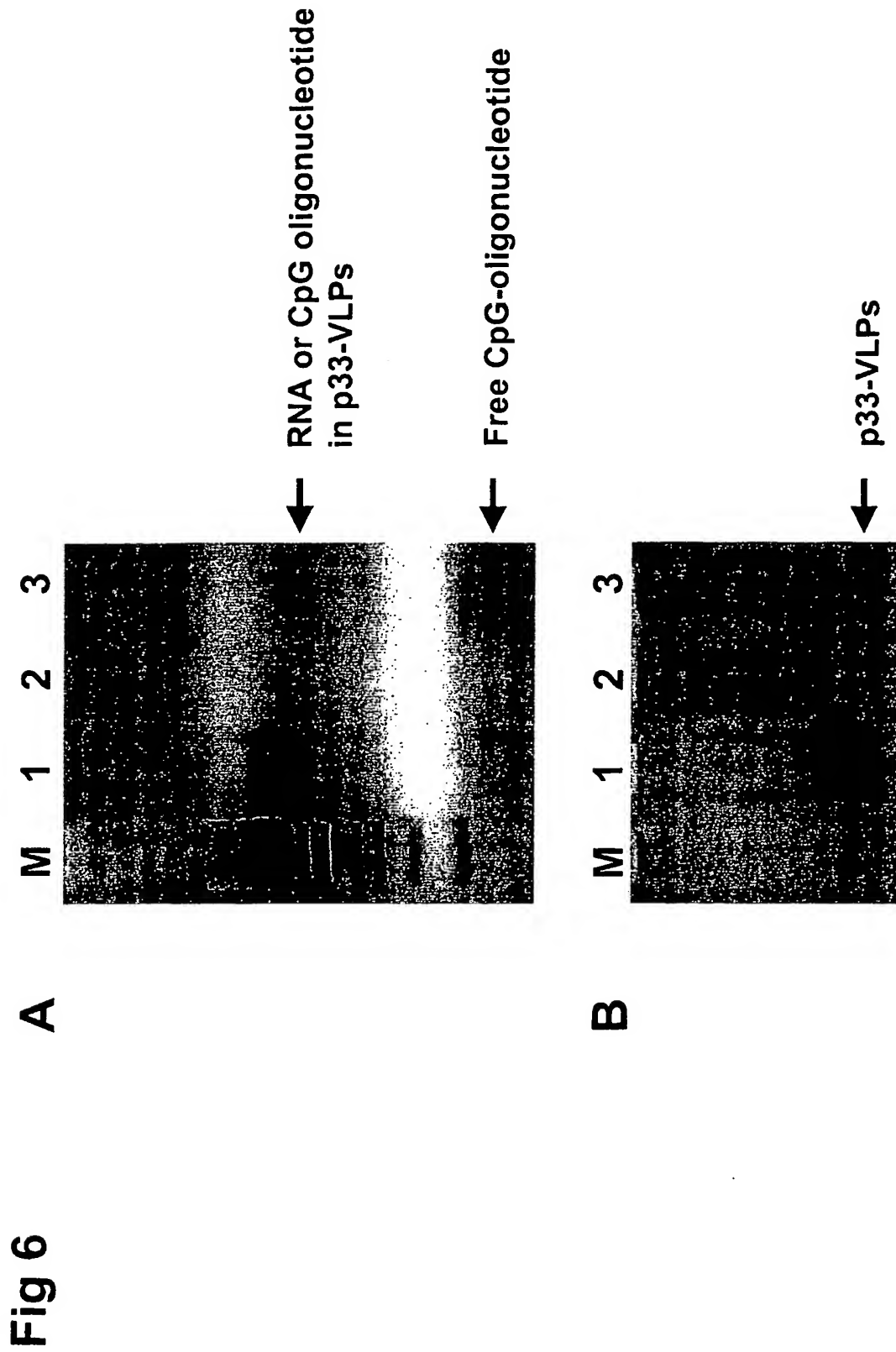
Fig. 2











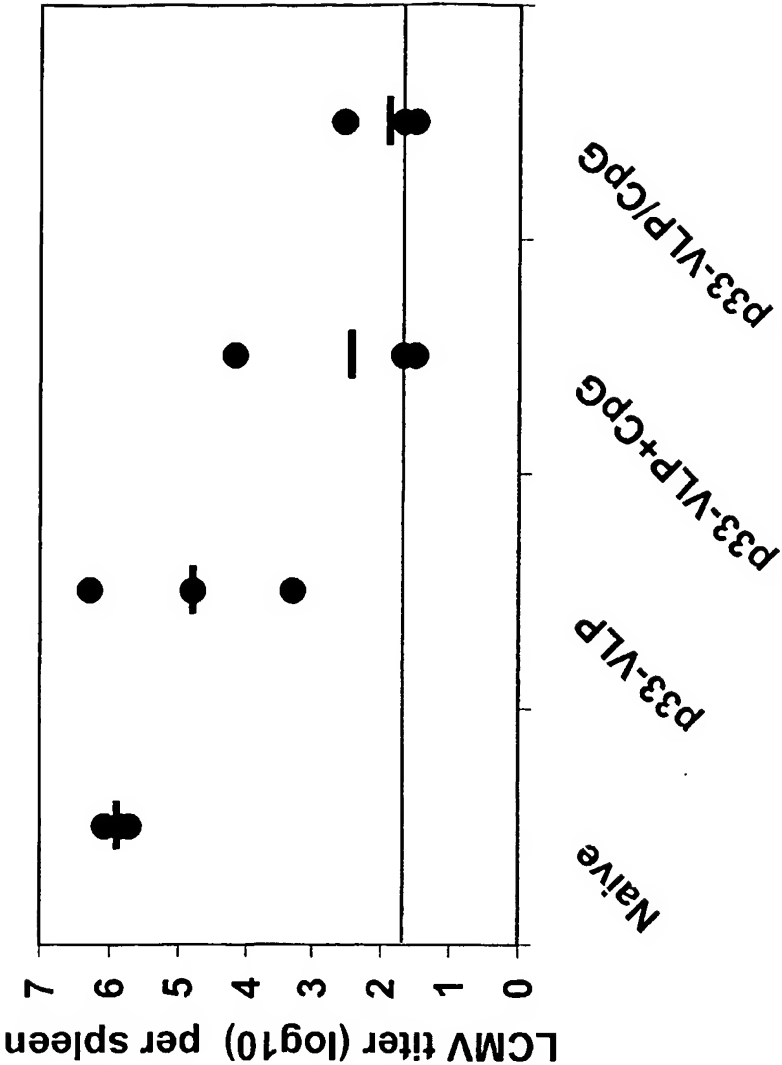


Fig 7



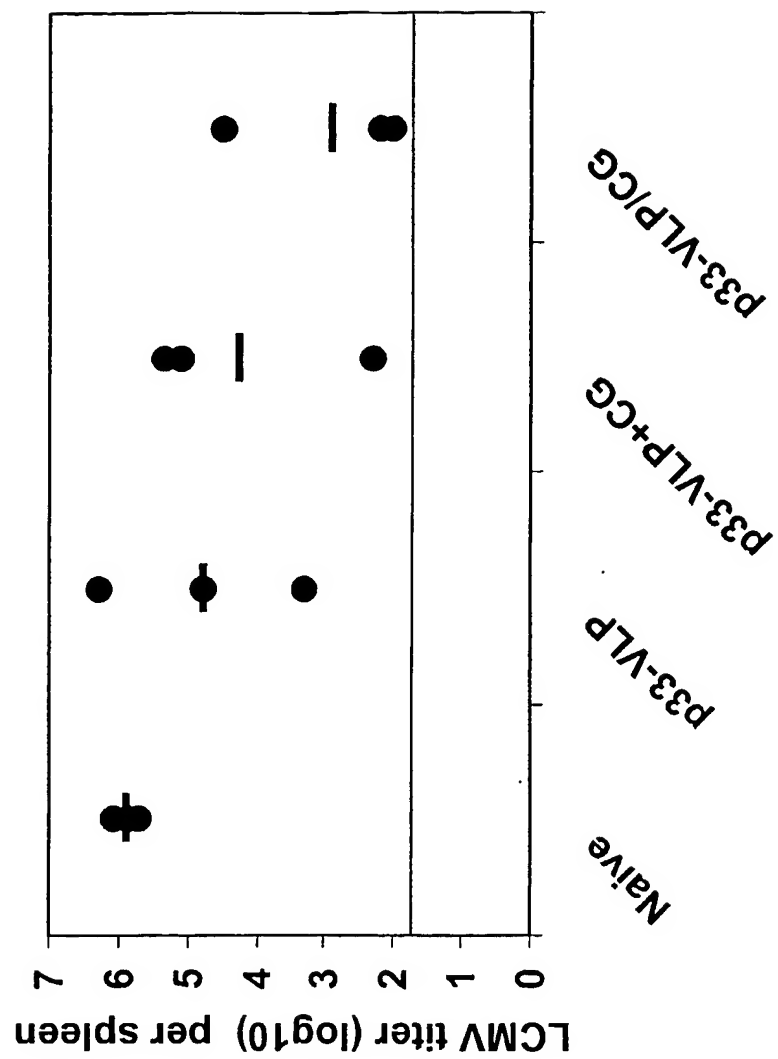


Fig 8

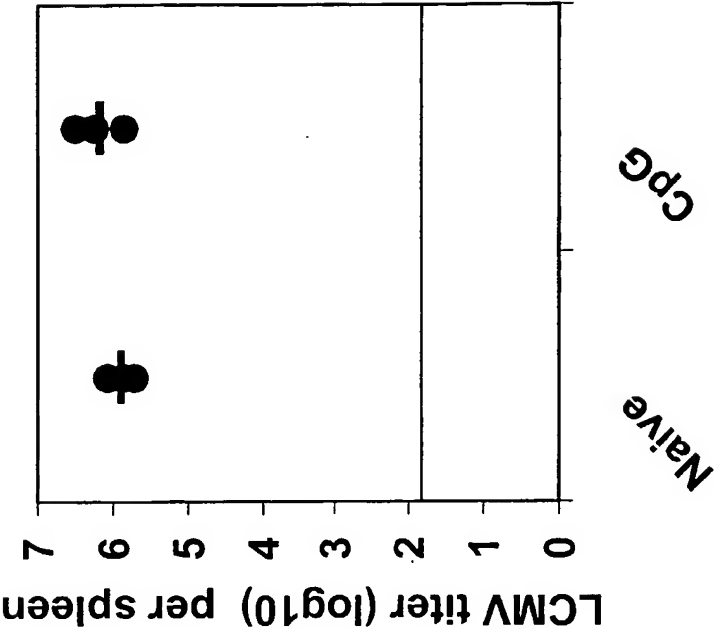


Fig 9

Fig. 10  
amino acid sequence of the BKV AS VP1 protein (GI:332779)

MAPTKRKGECPGAAPKKKEPVQVPKLLIKGGVEVLEVKTGVDAIT  
EVECFLNPEMGDPDDNLRGYSQHLSAENAFESDSPDRKMLPCYSTA  
RIPLPNLNEDLTCGNLLMWEAVTVKTEVIGITSMNLNLHAGSQKVHE  
NGGKPVQGSNFHFFAVGGDPLEMQGLMNYRTKYPQGTITPKNPT  
AQSQVMNTDHKAYLDKNNAYPVECWIPDPSRNENTRYFGTYTGGEN  
VPPVLHVTNTATTVLLDEQQGVGPLCKADSLYVSAADICGLFTNSSG  
TQQWRGLARYFKIRLRKRSVKNPYPI SFLLSDLINRRTQKVVDGQPM  
YGMESQVEEVRFVDGTEQLPGDPPMIRYIDRQQQLQTKMV

Fig. 11  
nucleic acid sequence of the 246 bp DNA fragment

```
1   GCGGGTGGTG TCAGATCTAC AATGATCGTC ATCACCTTGG TGATGCTGAA
51  GAAGAAACAG TACACATCCA TTCATCATGG TGTGGTGGAG GTTGACGCCG
101 CTGTCACCCC AGAGGAGCGC CACCTGTCCA AGATGCAGCA GAACGGCTAC
151 GAAAATCCAA CCTACAAGTT CTTTGAGCAG ATGCAGAACG CTAGCTATCC
201 ATACGATGTC CCTGATTACG CCTAACGCCA ATTGCCCAGC ACAGTG
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Fig. 12

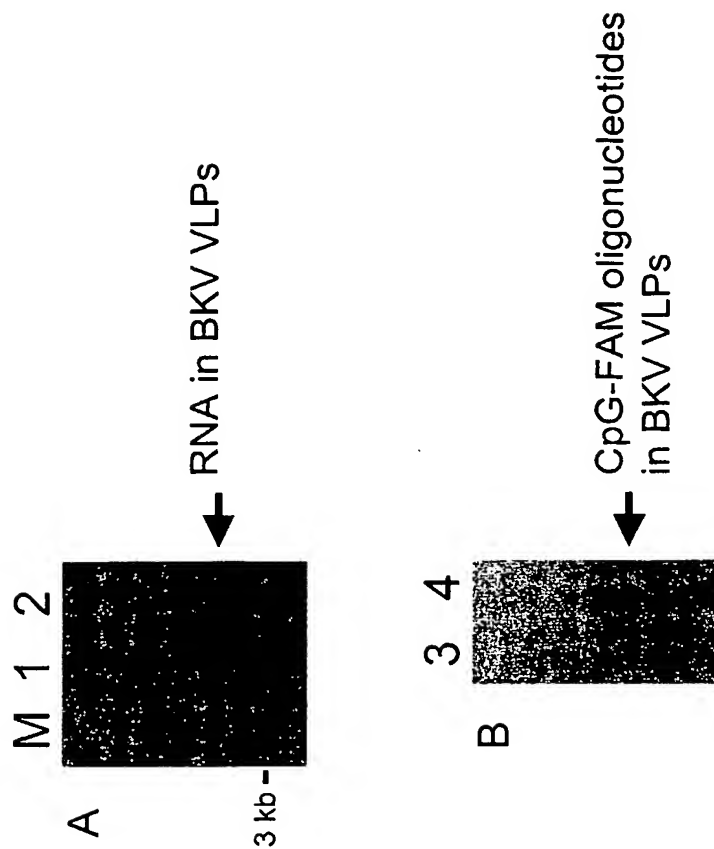
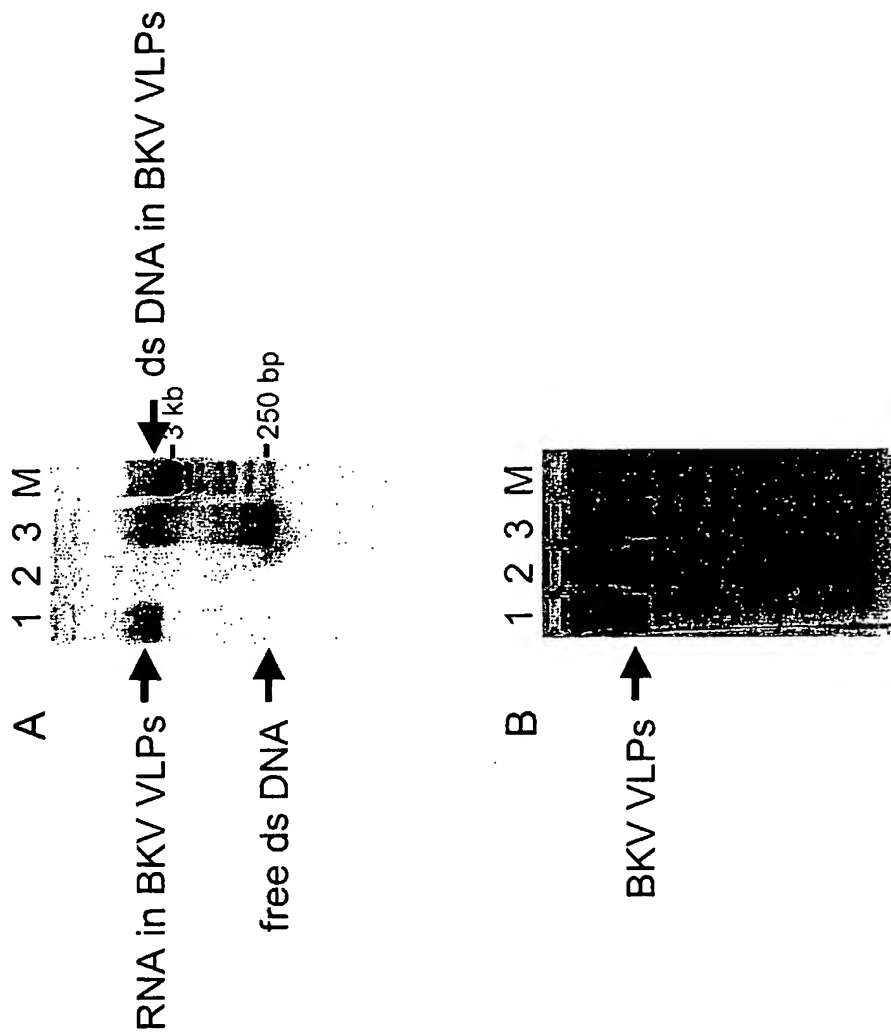


Fig. 13



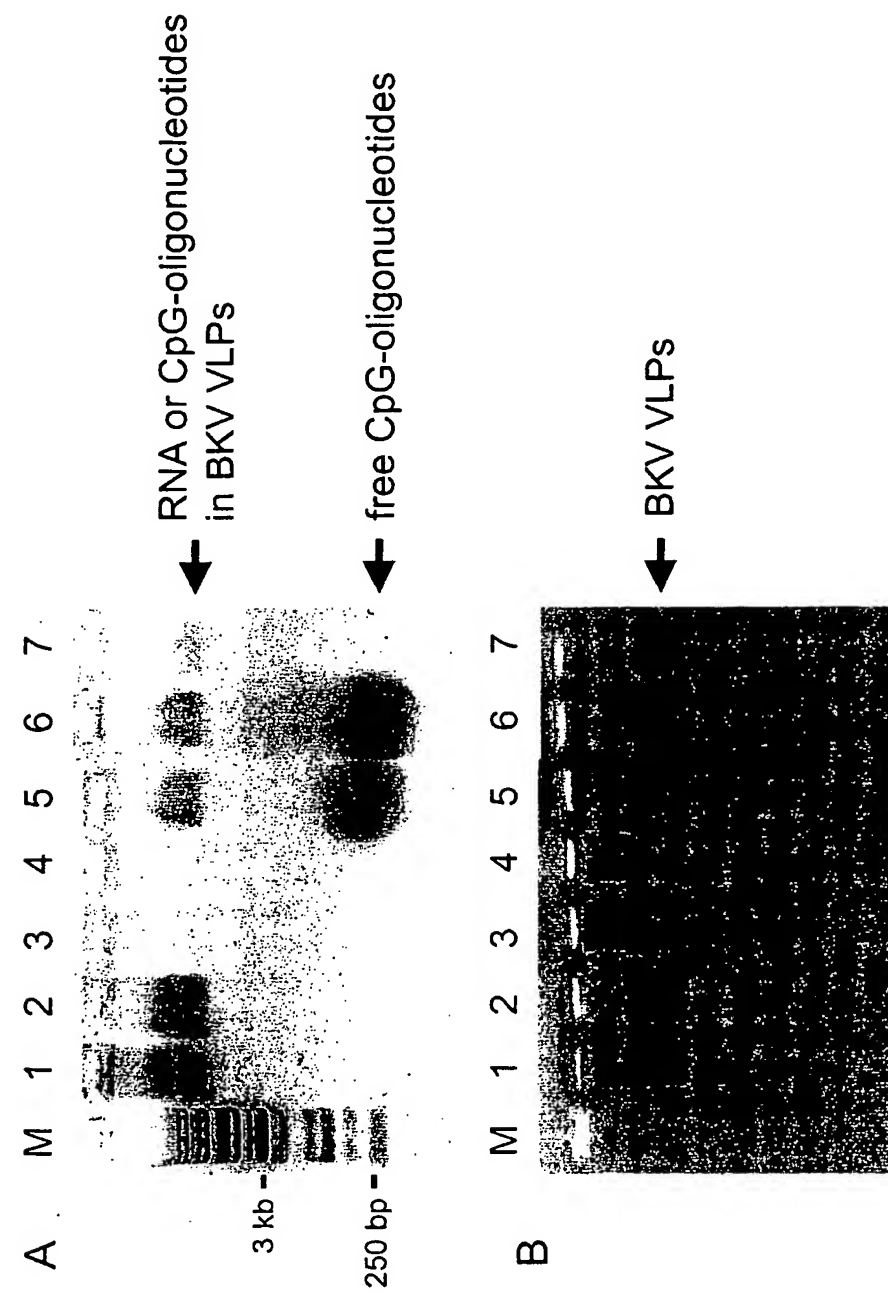
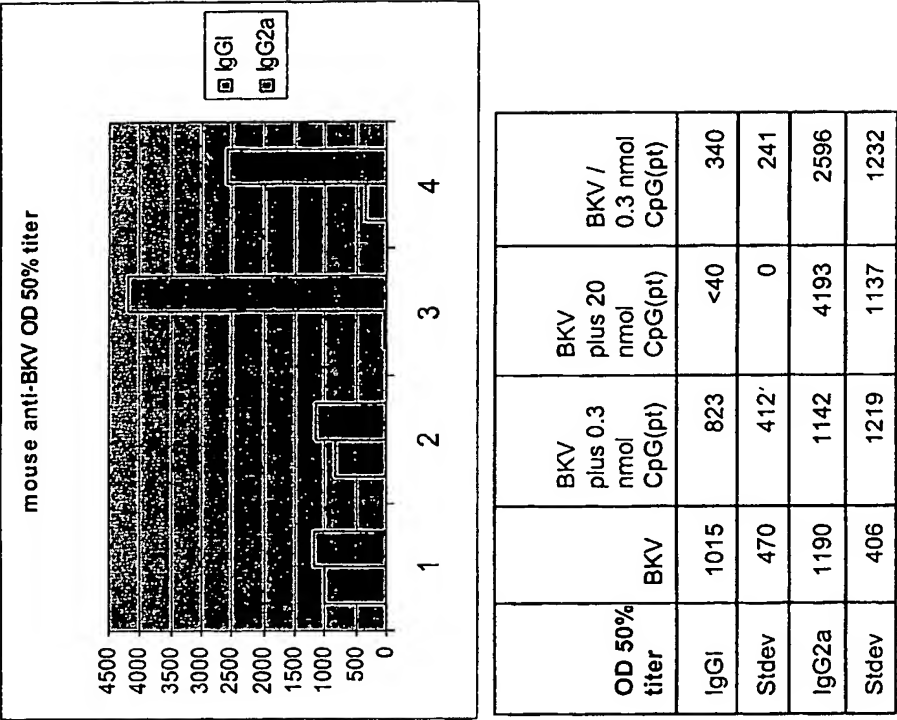
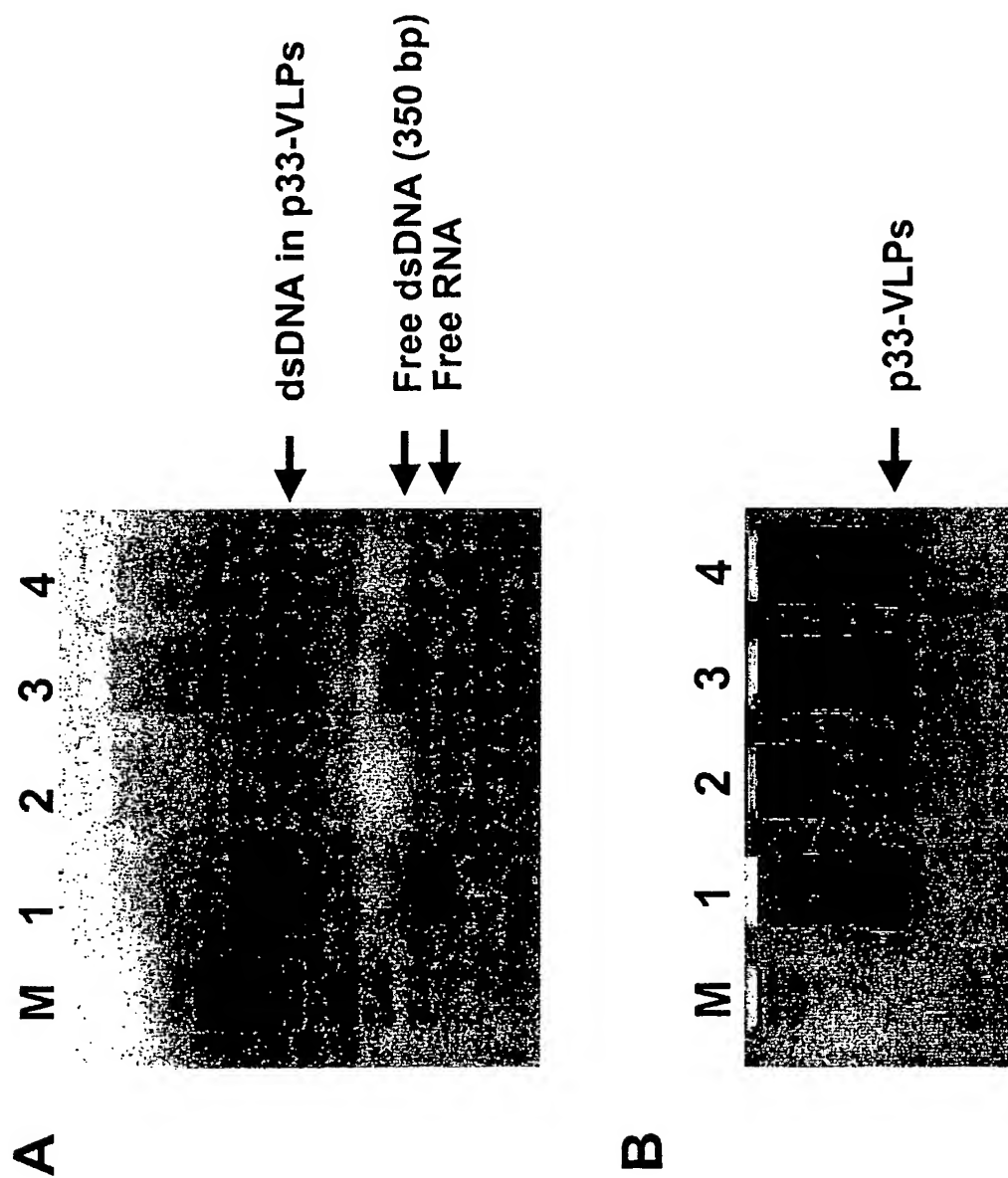


Fig. 15





**Fig. 16**



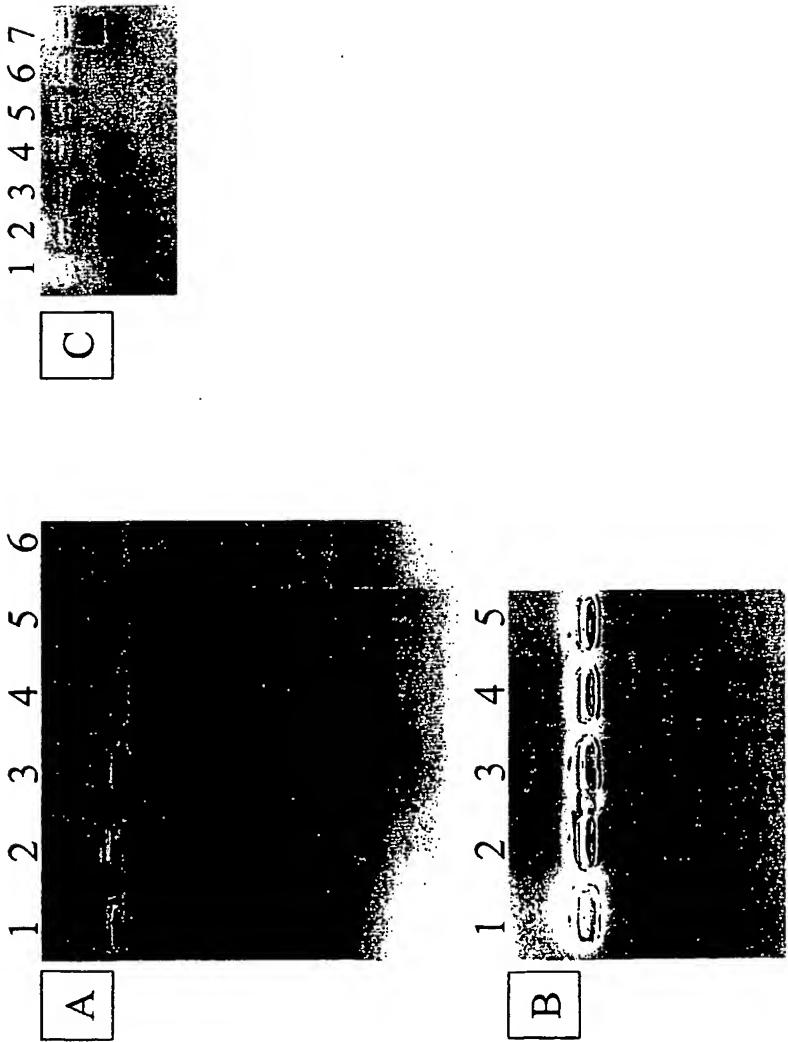


FIG. 17

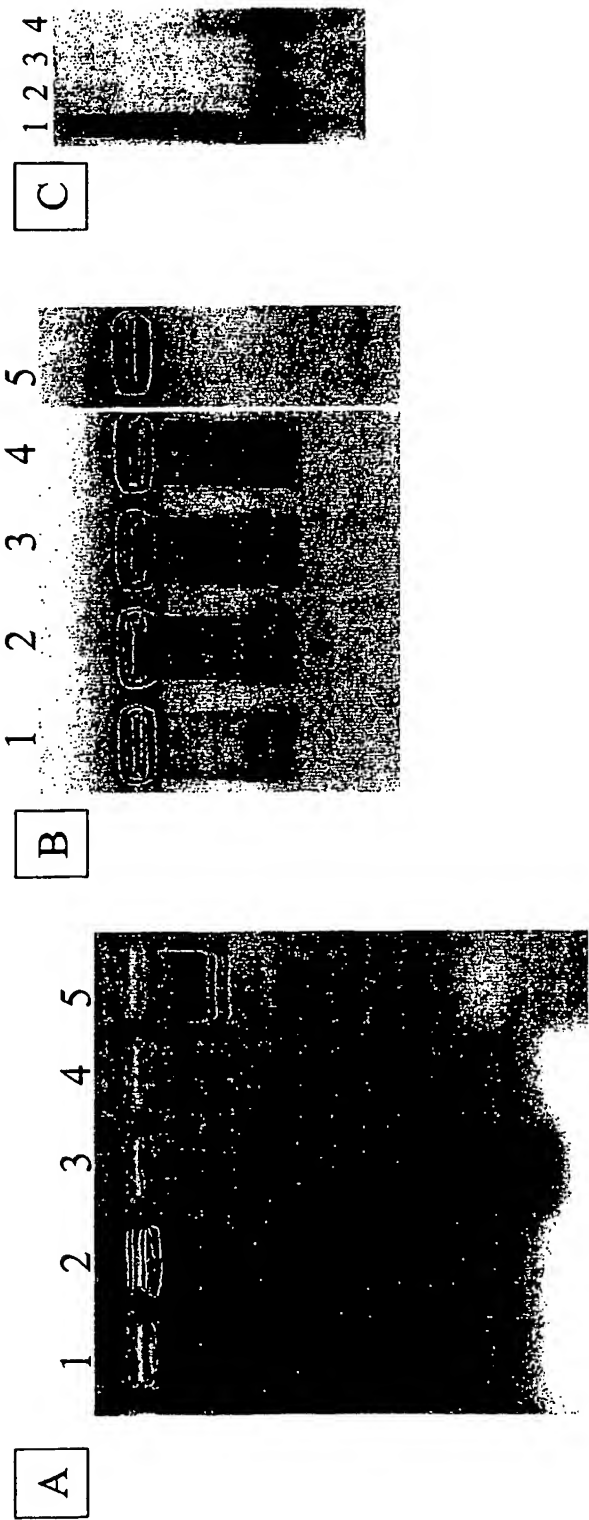


FIG. 18

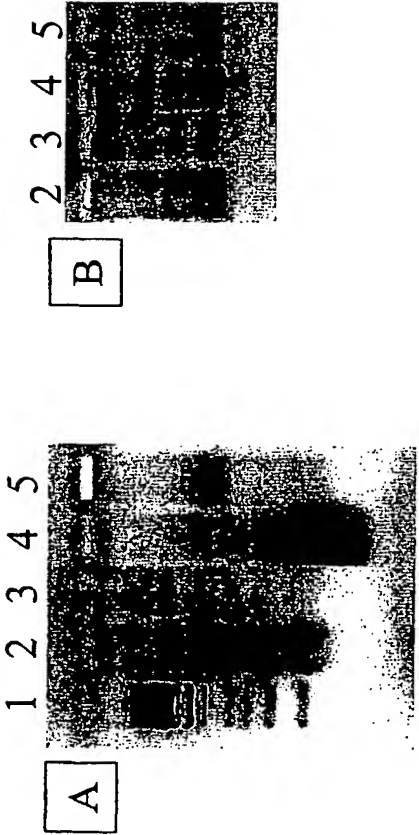


FIG. 19

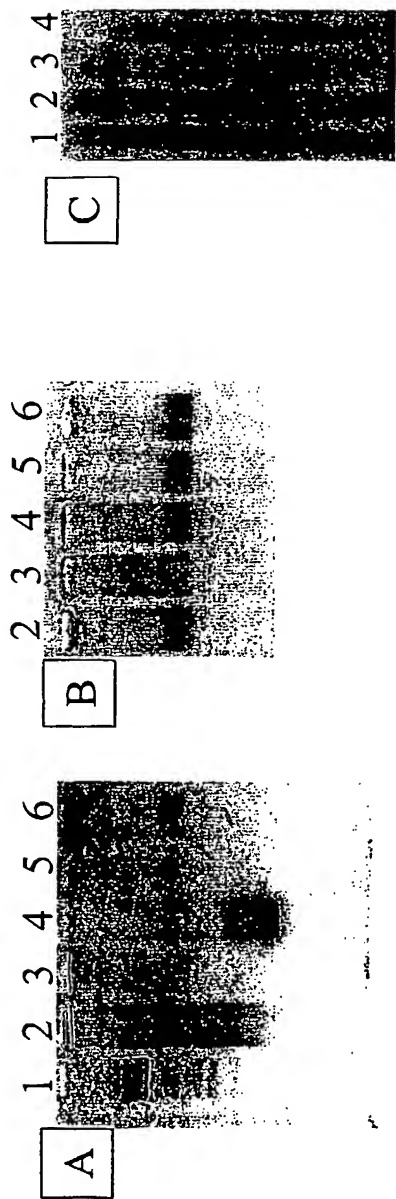


FIG. 20

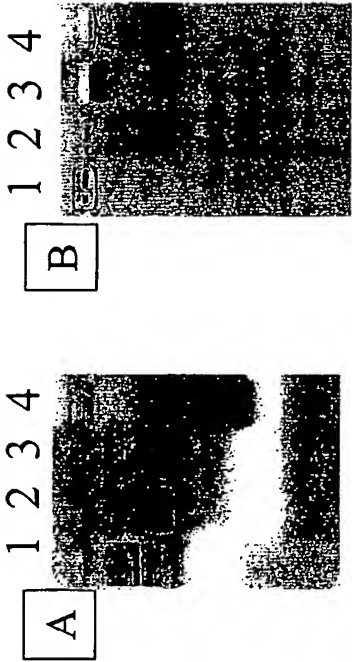


FIG. 21

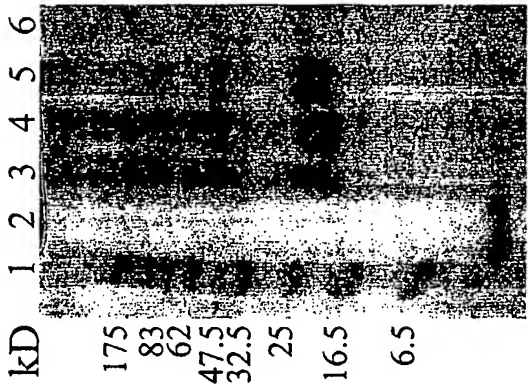


FIG. 22

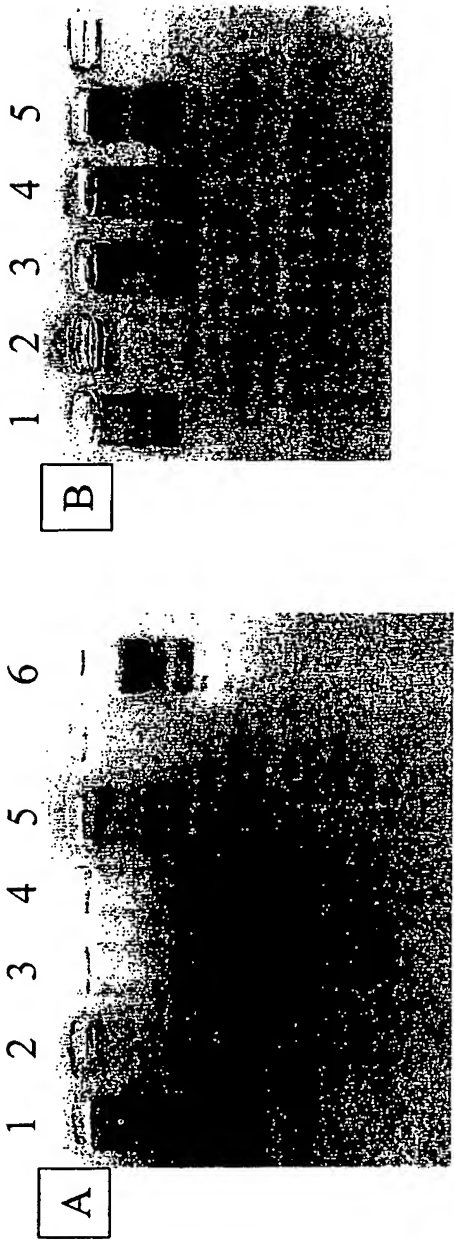


FIG. 23



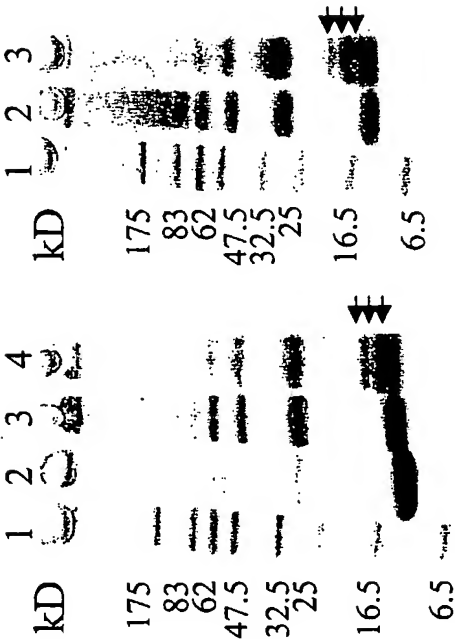


FIG. 24

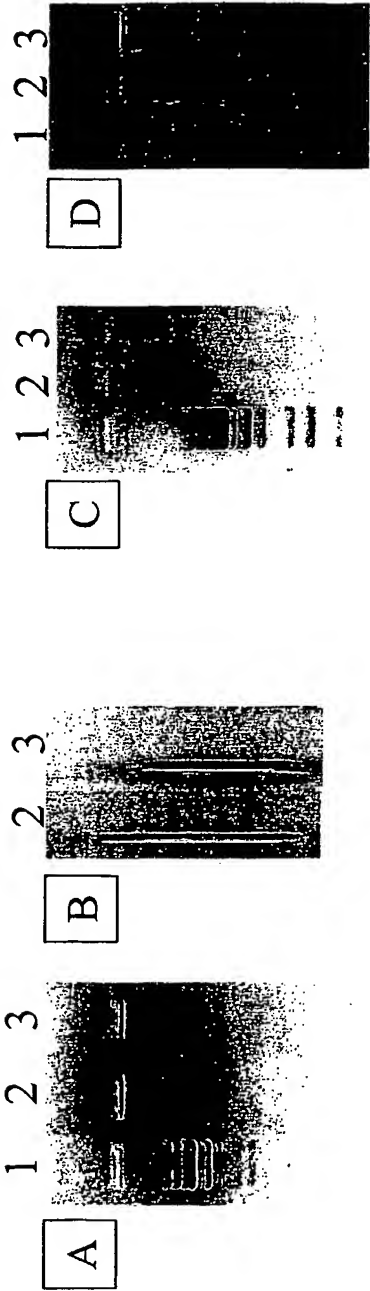


FIG. 25

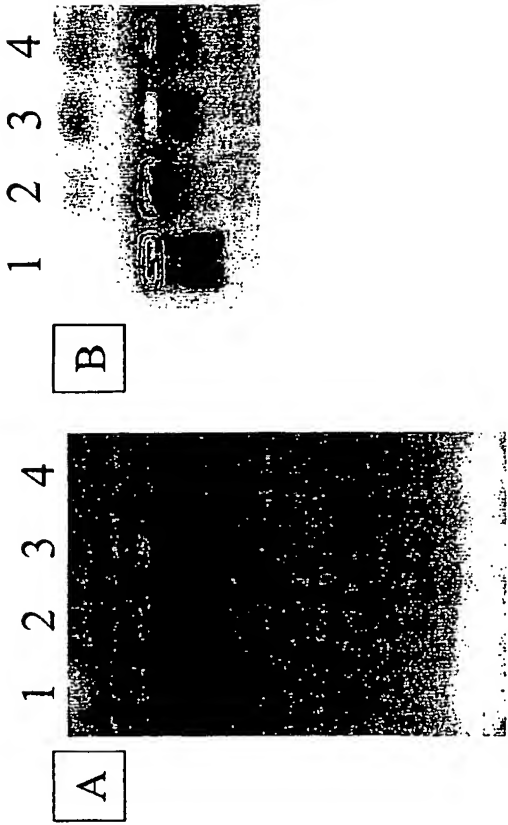


FIG. 26

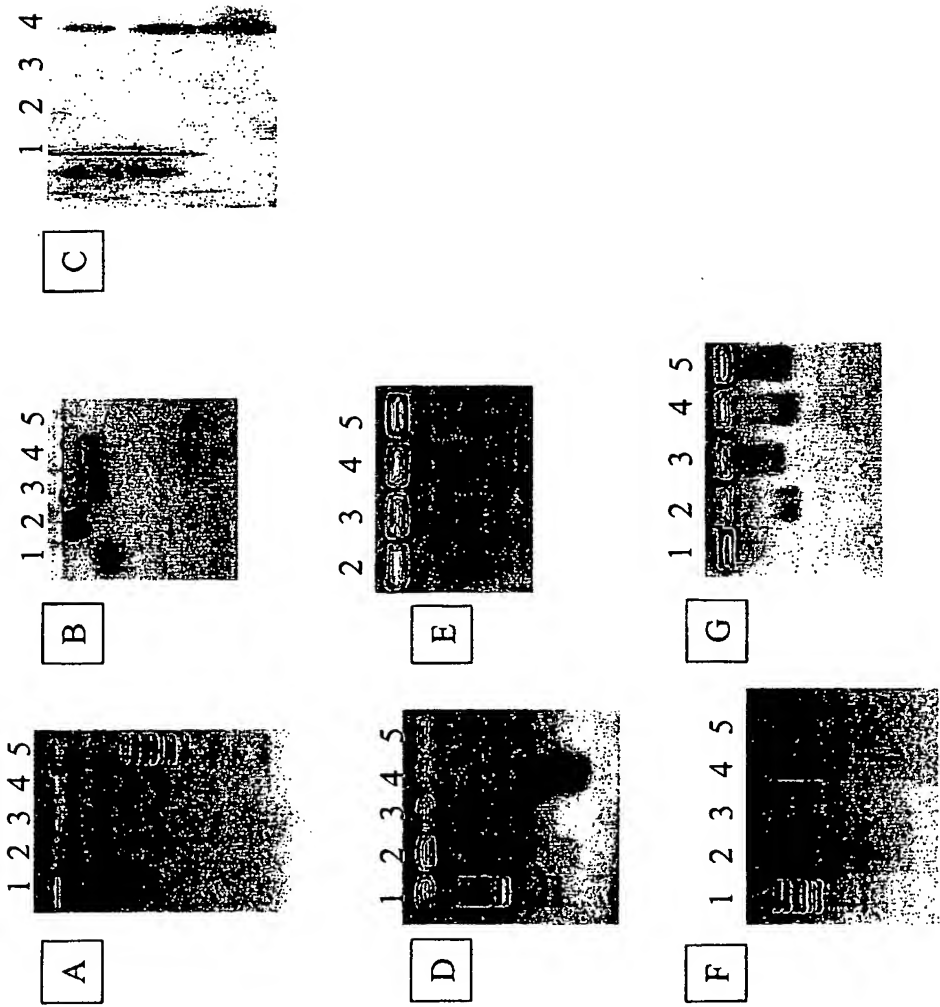
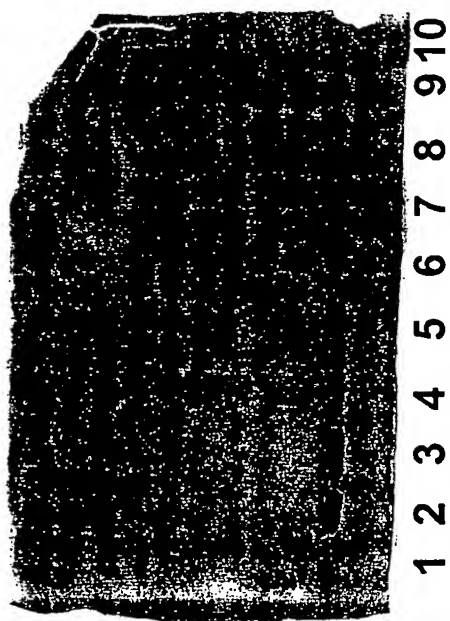
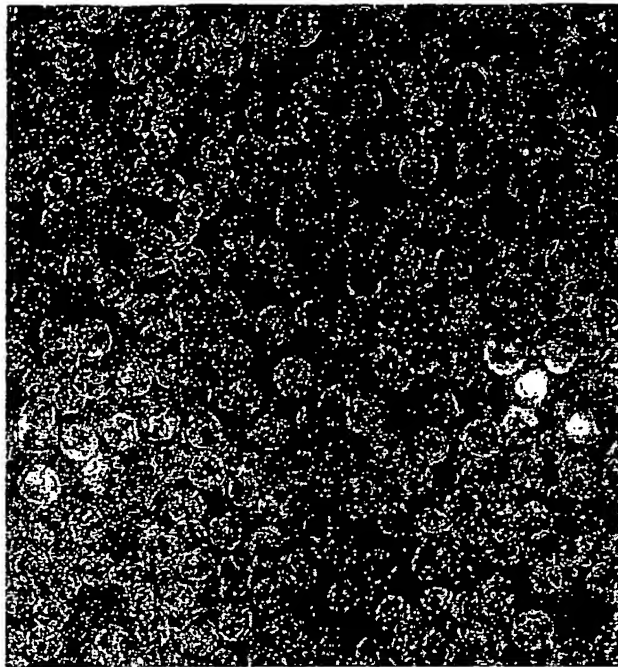


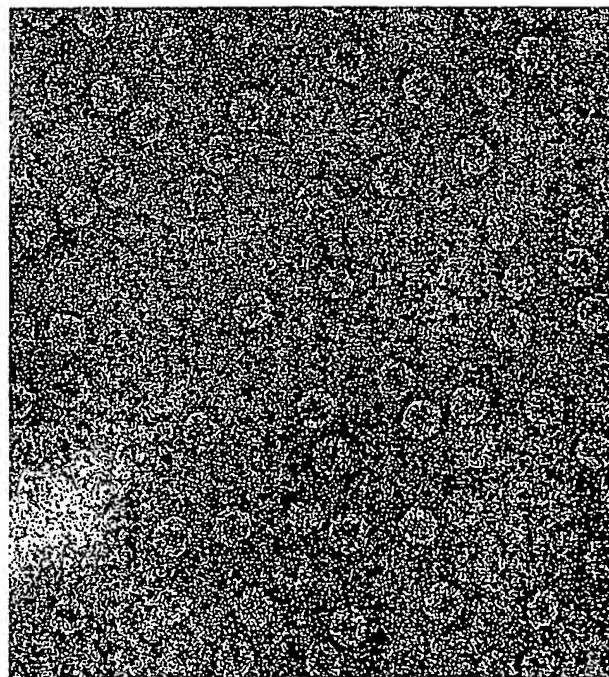
FIG. 27



**FIG. 28**



**B**



**A**

**FIG. 29**

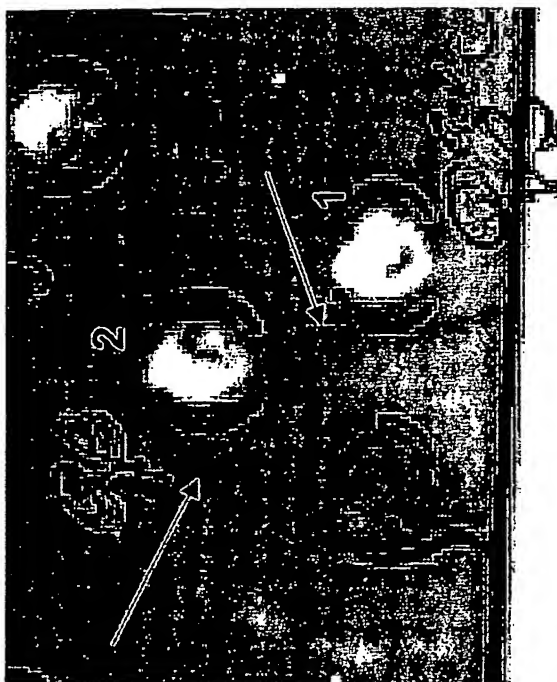


Fig. 30 A

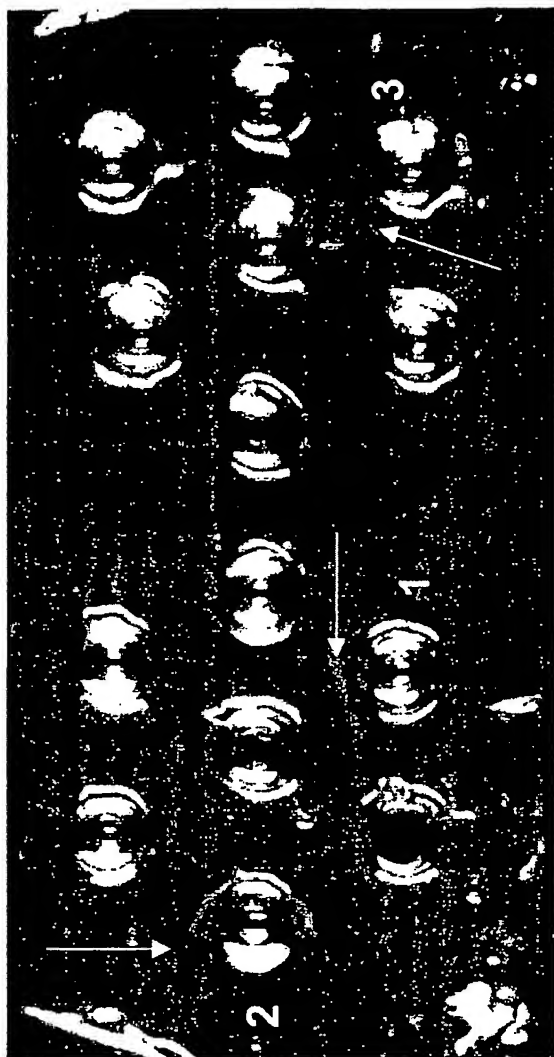


Fig. 30 B



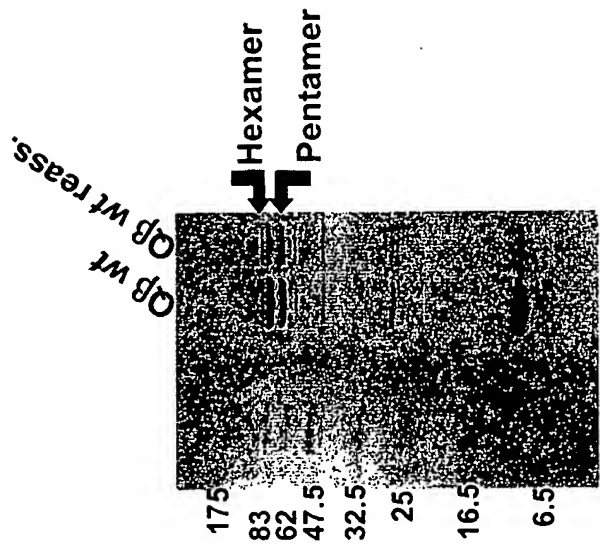


Fig. 31 A

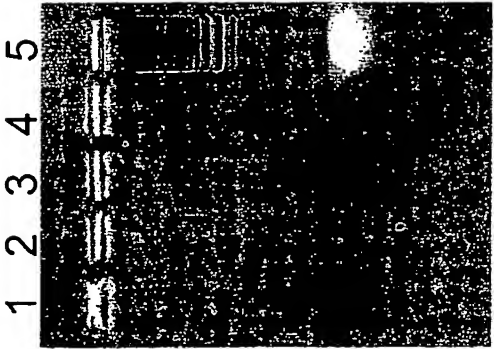


Fig. 31 B

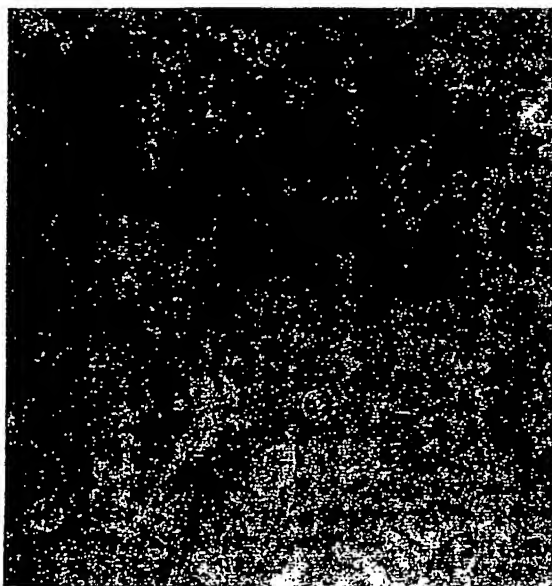


Fig. 32

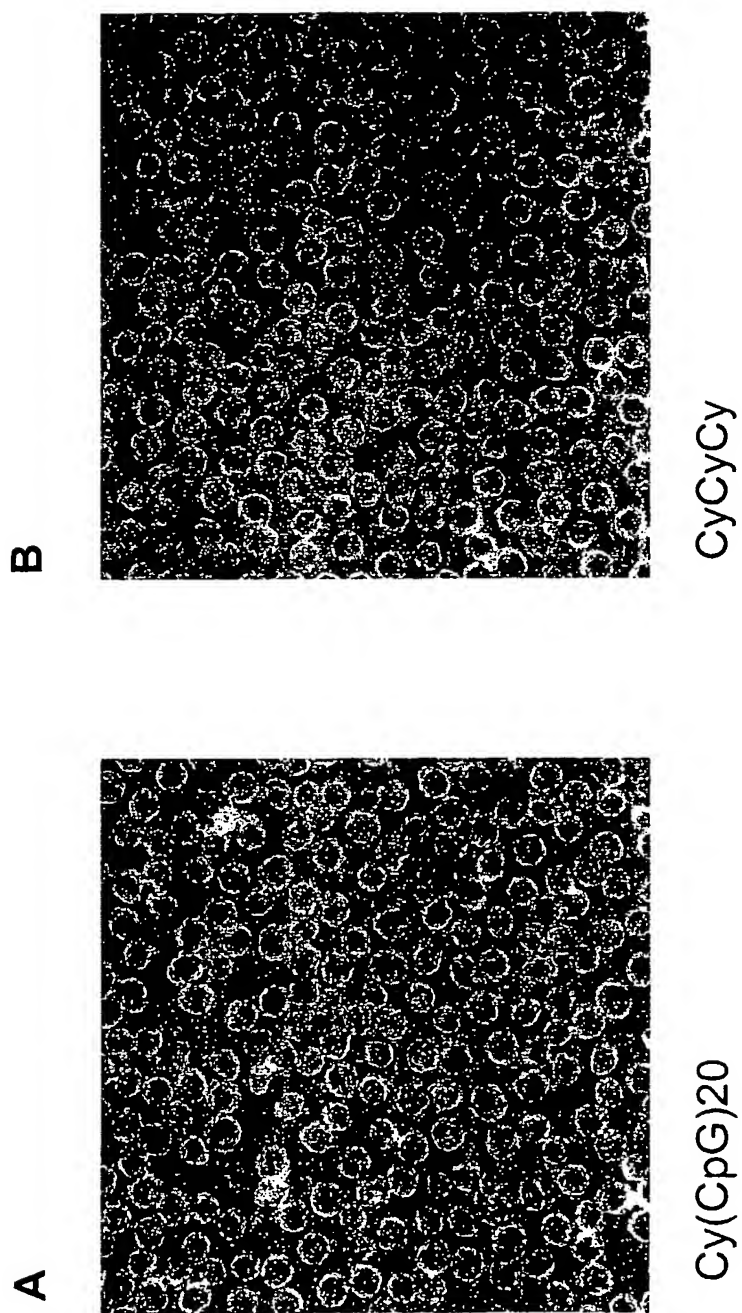


Fig. 33

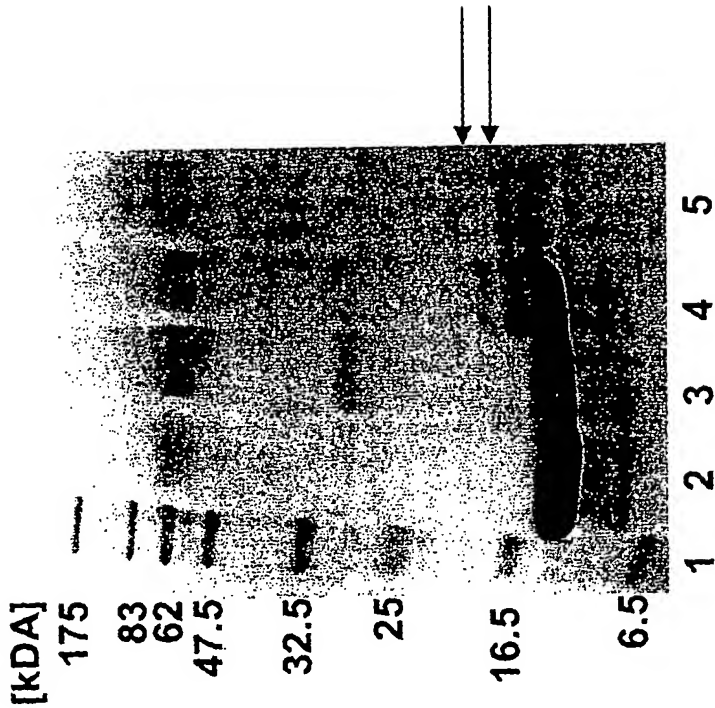


Fig. 34

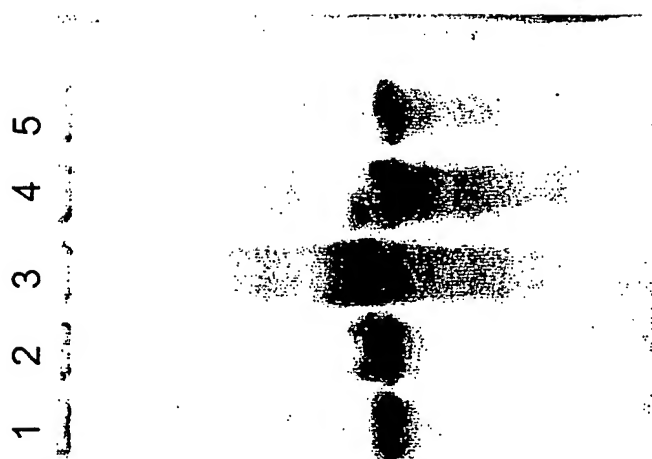


Fig. 35

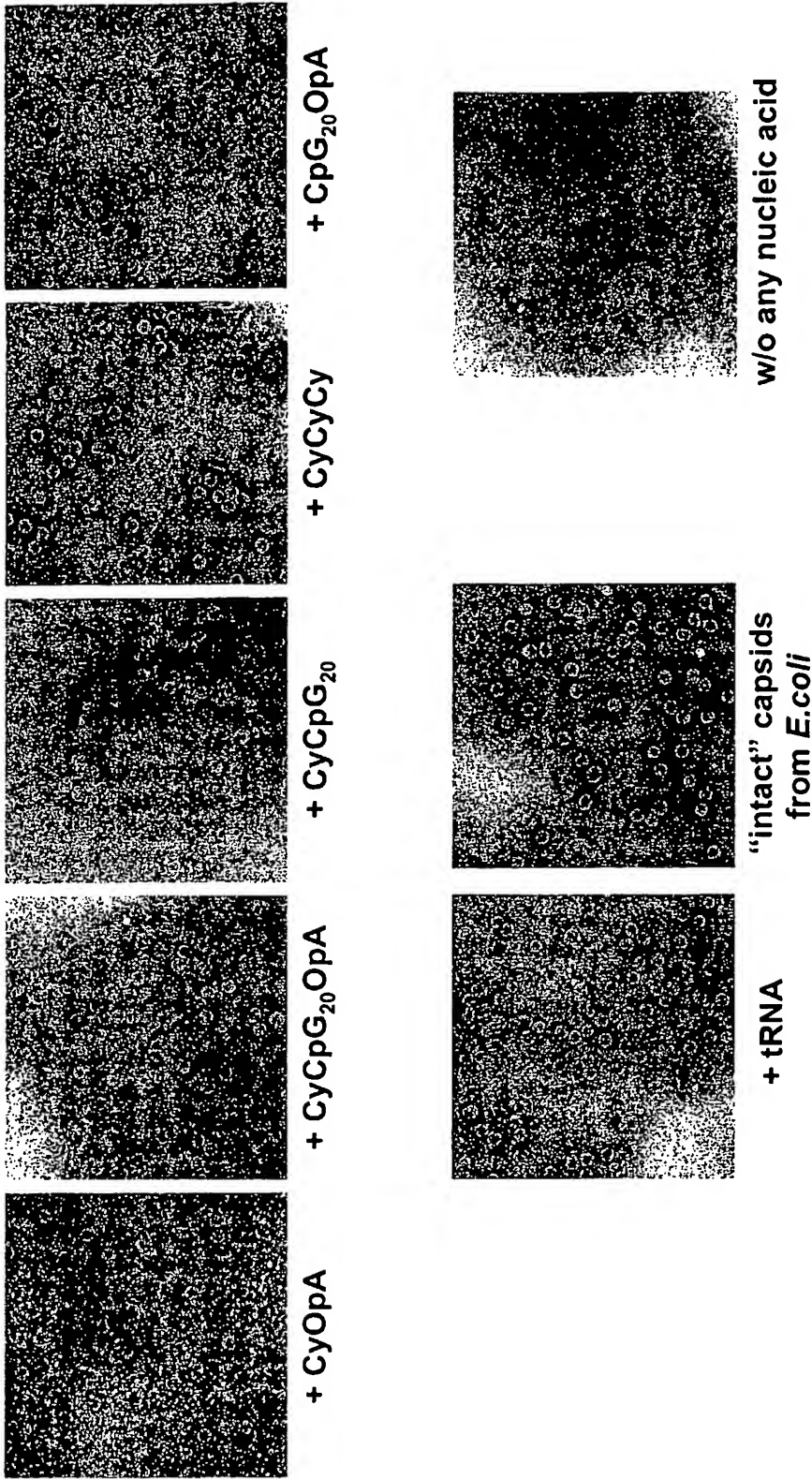
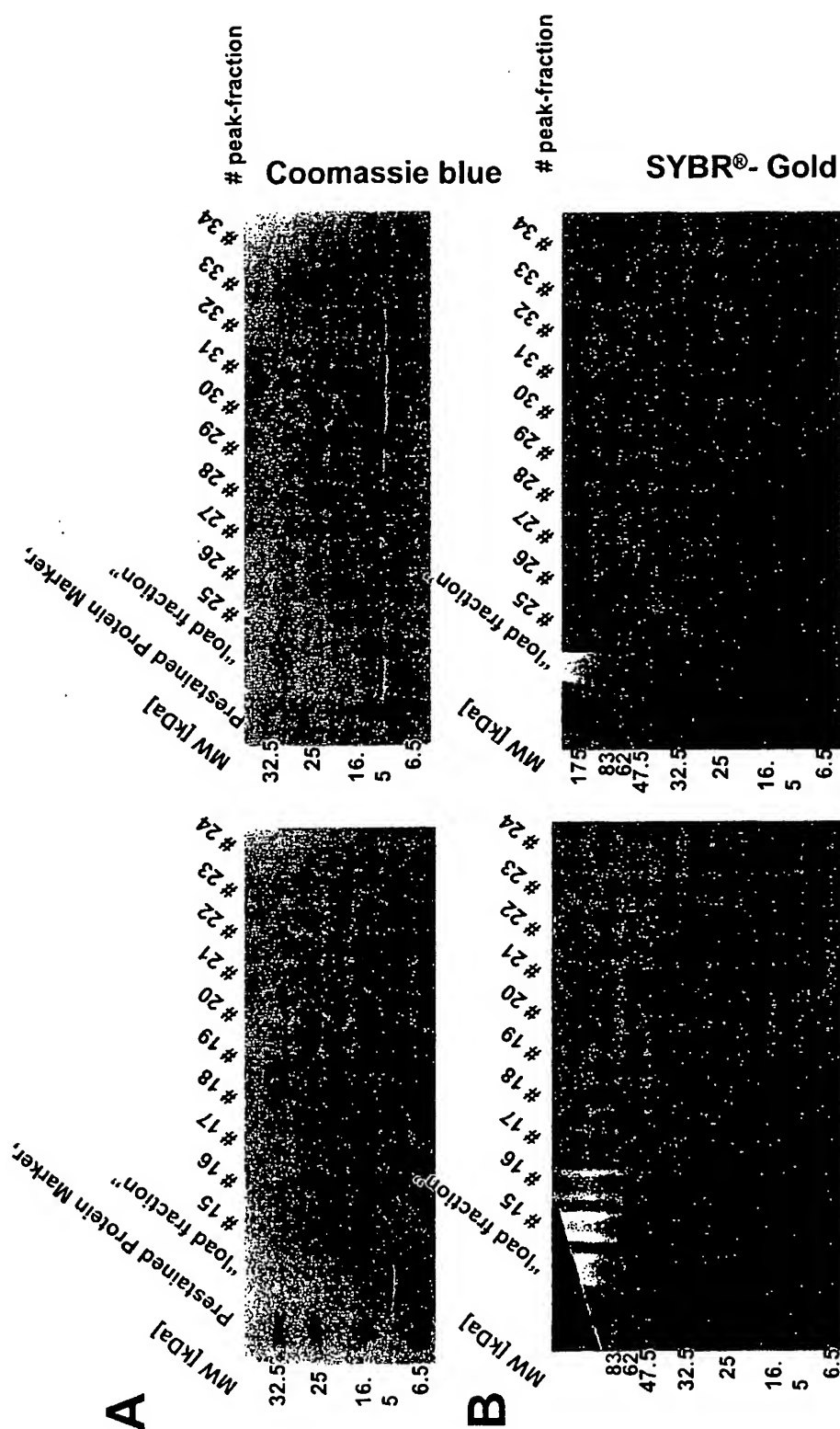


Fig. 36



**Fig. 37**



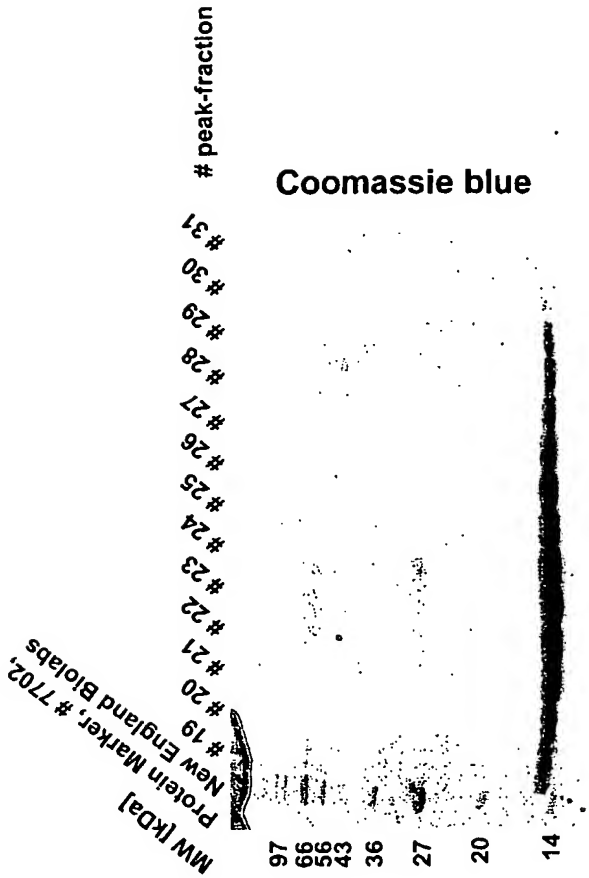


Fig. 38

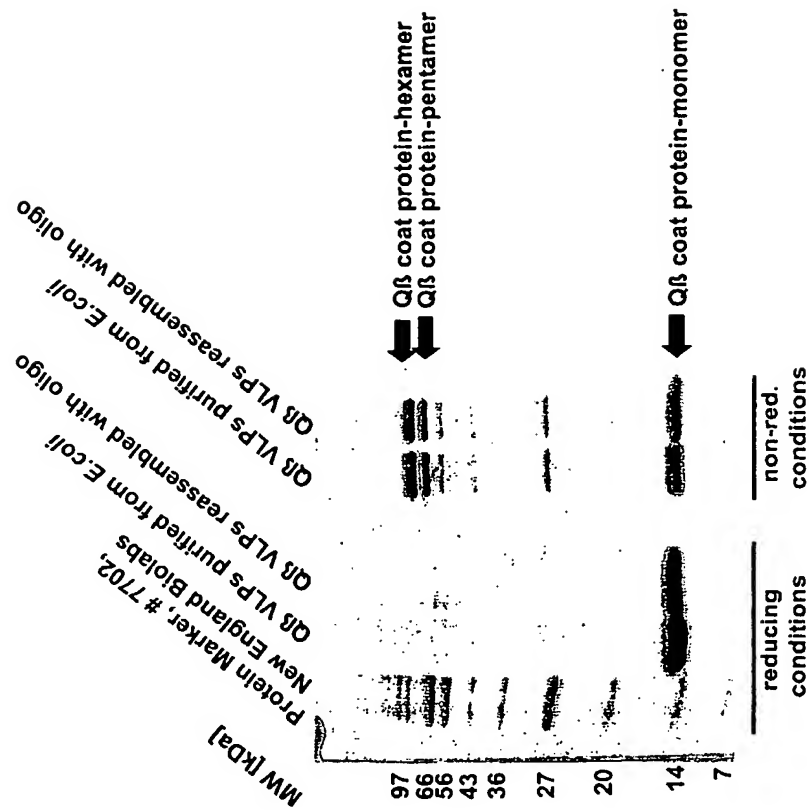
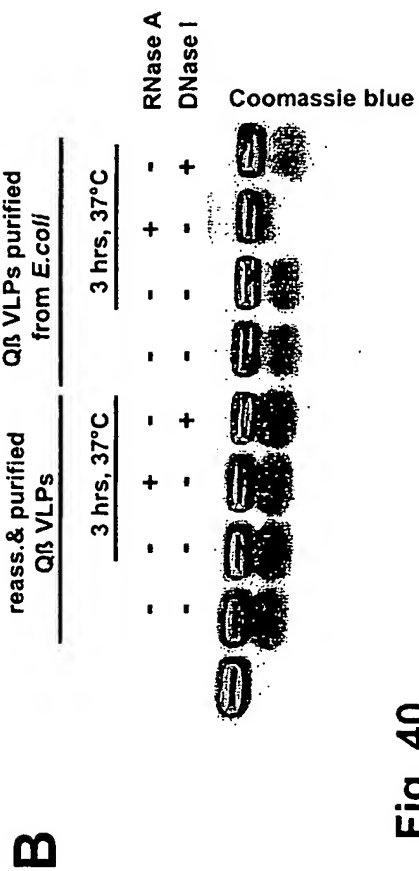
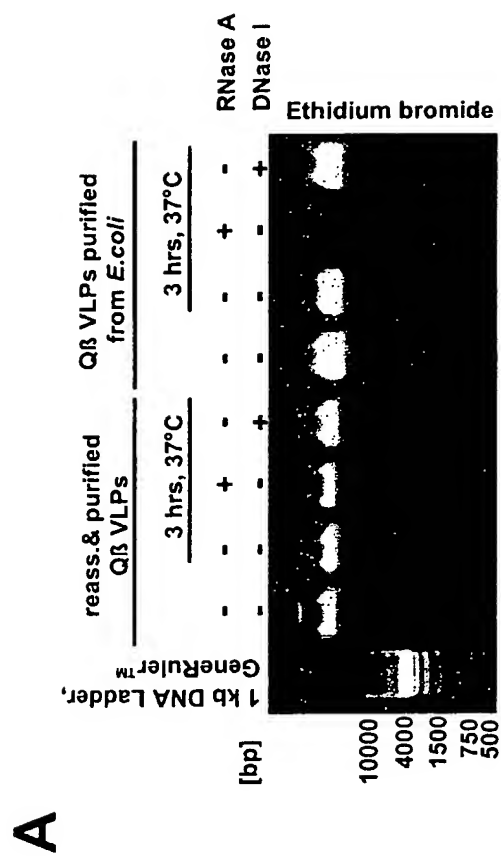


Fig. 39



**Fig. 40**

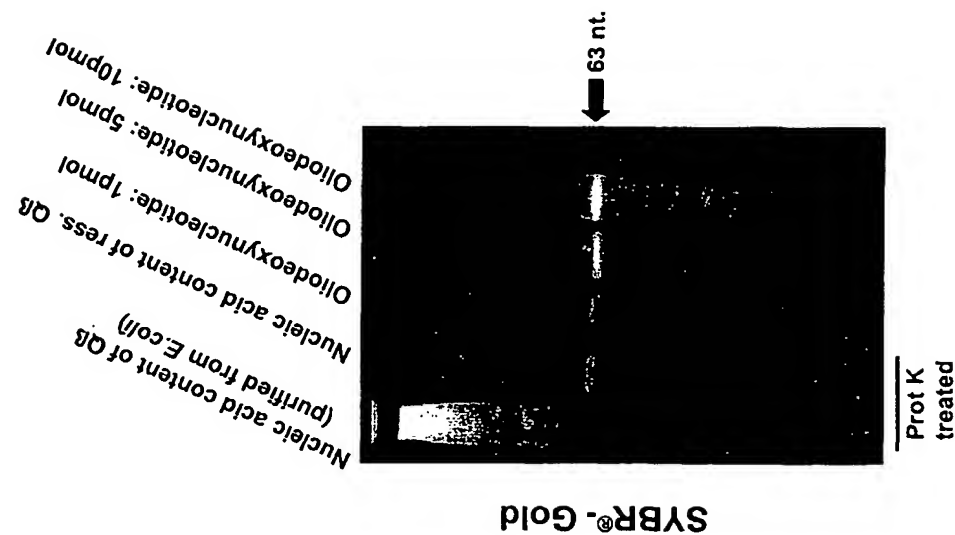
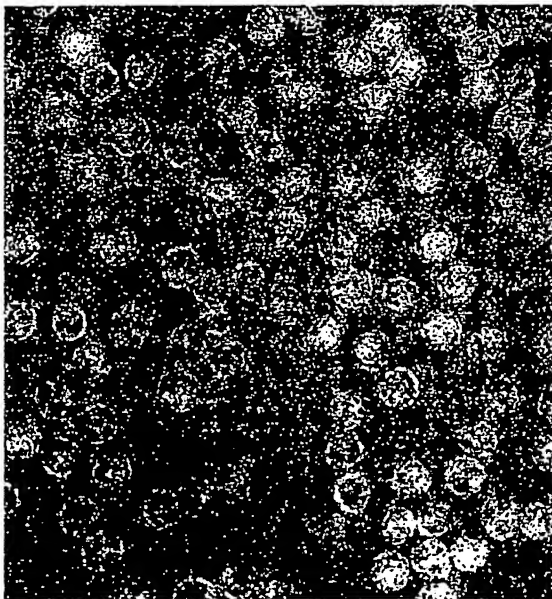


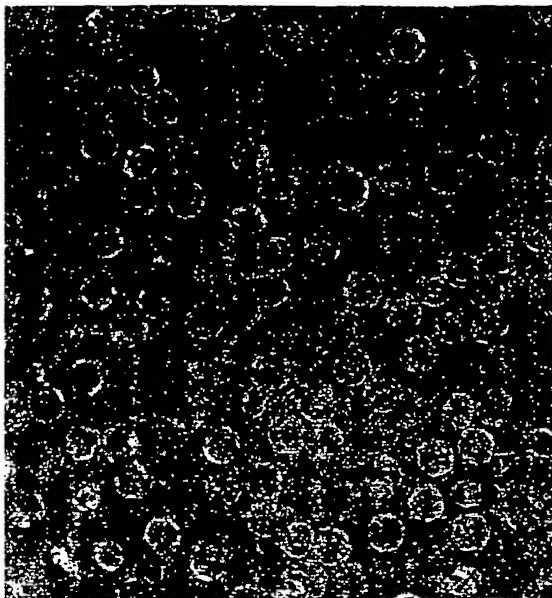
Fig. 41

**A**



recombinant

**B**



reassembled

**Fig. 42**

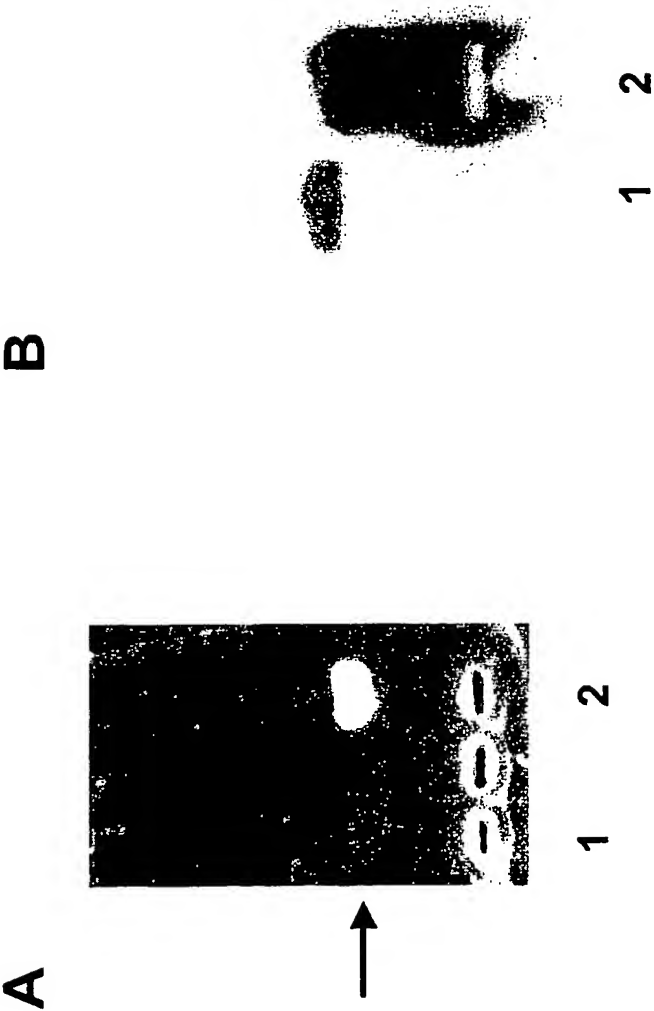
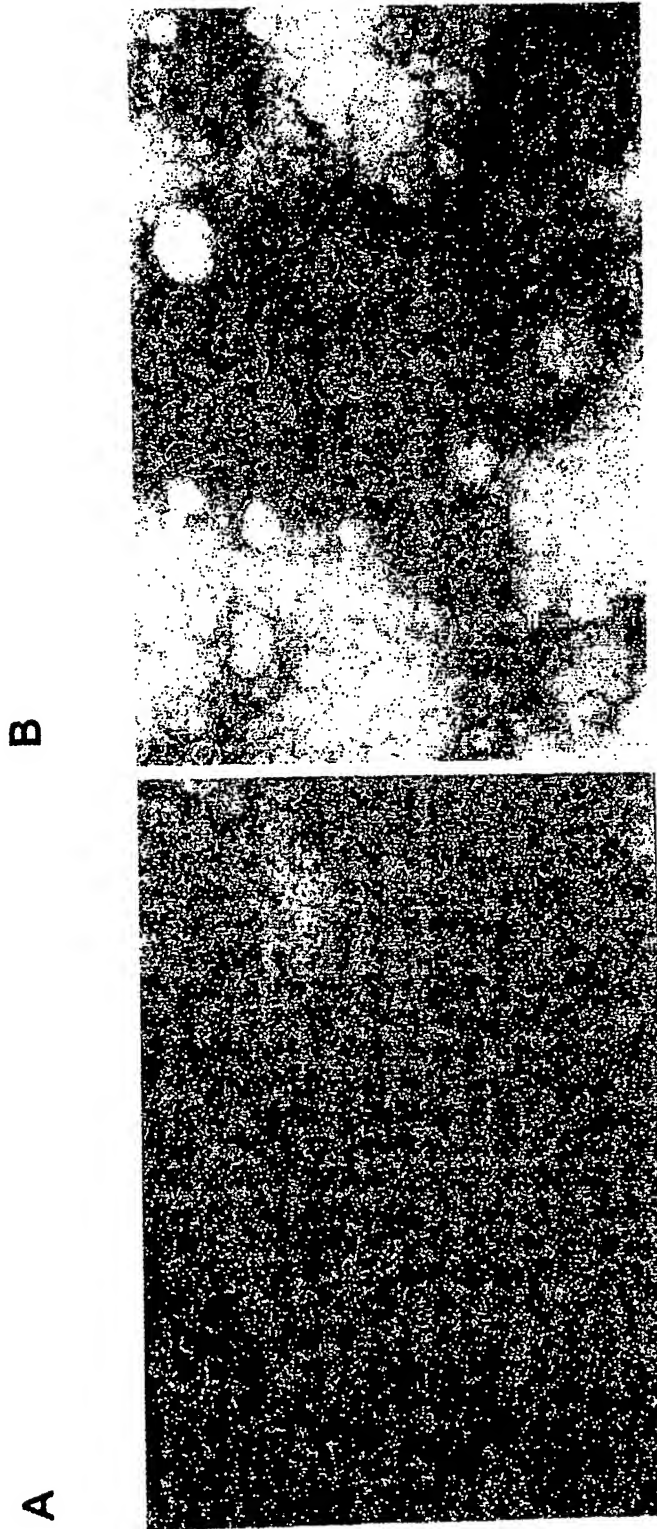


Fig. 43



**Fig. 44**

C

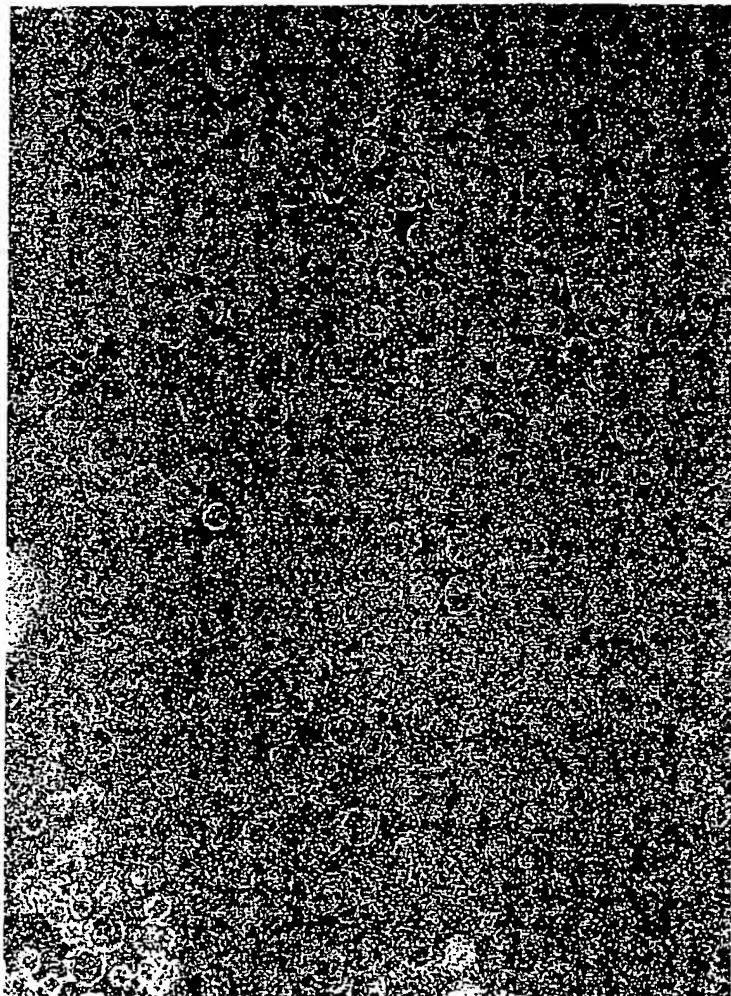


Fig. 44



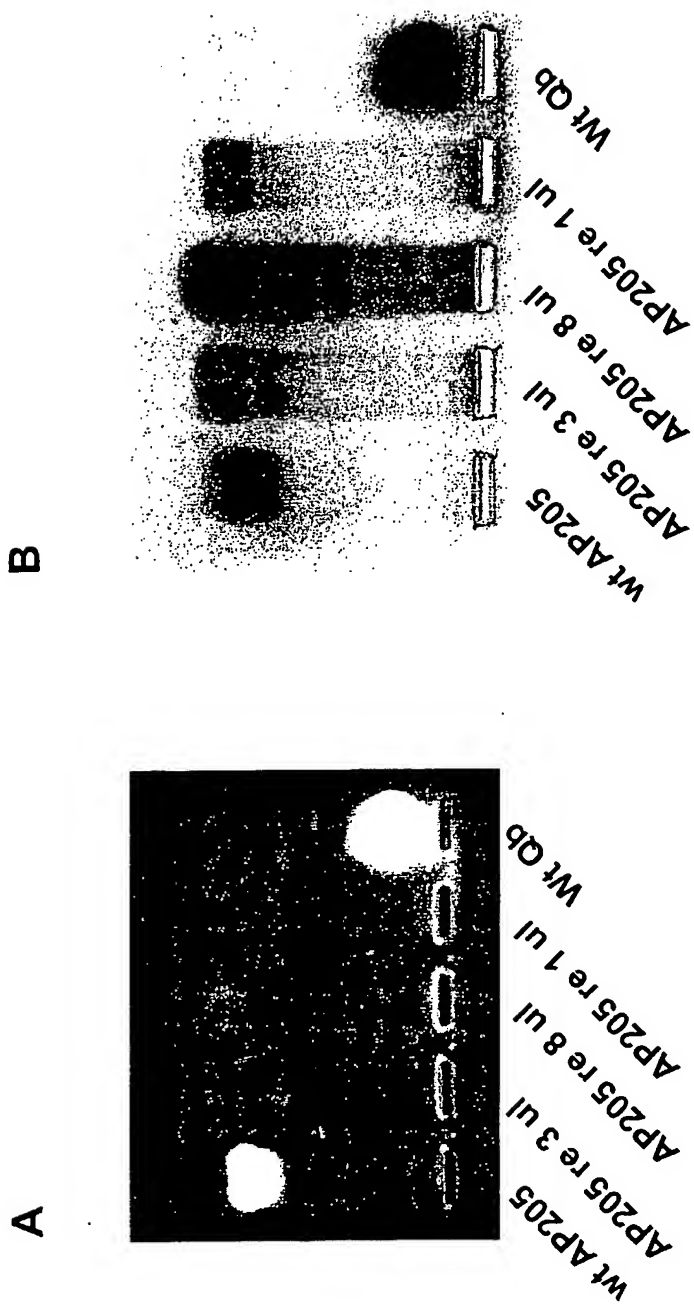


Fig. 45

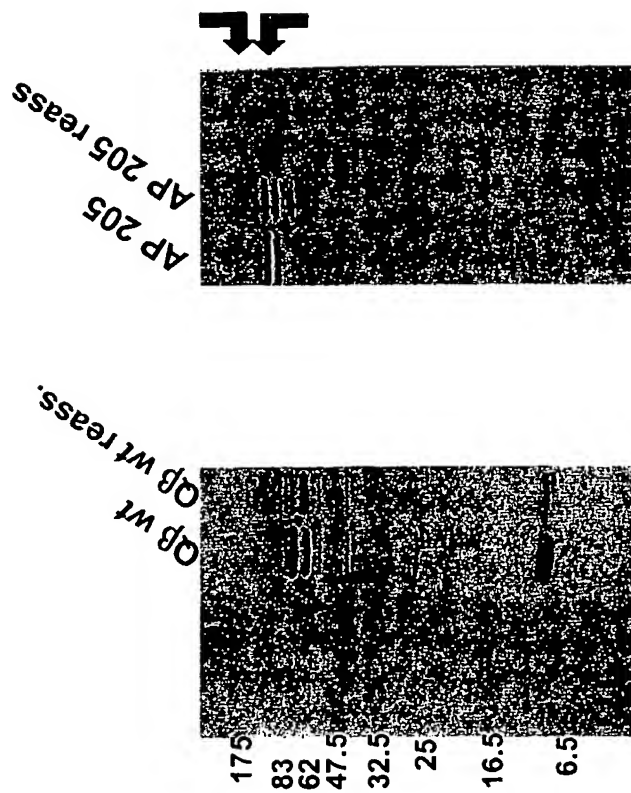


Fig. 46

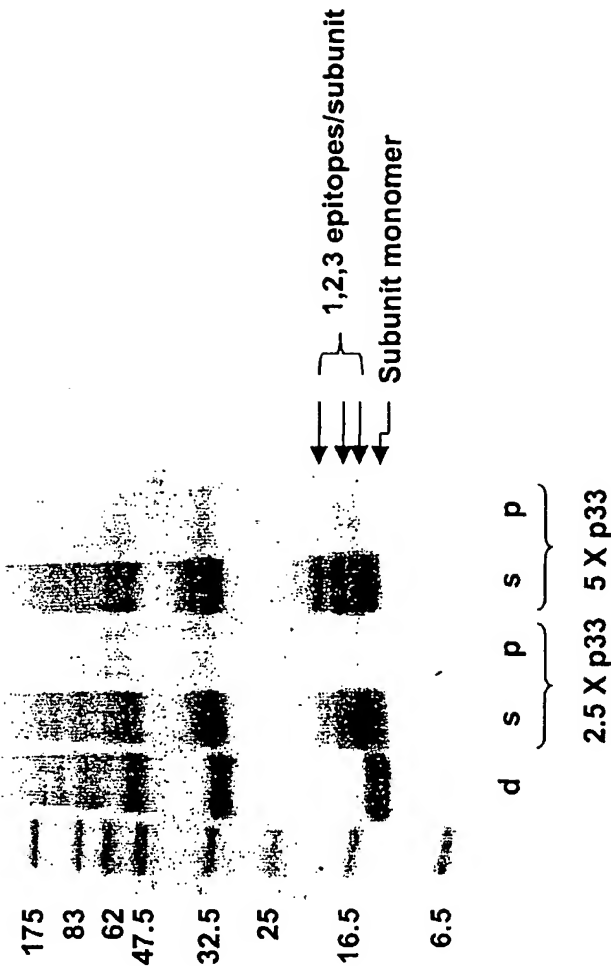


Fig. 47

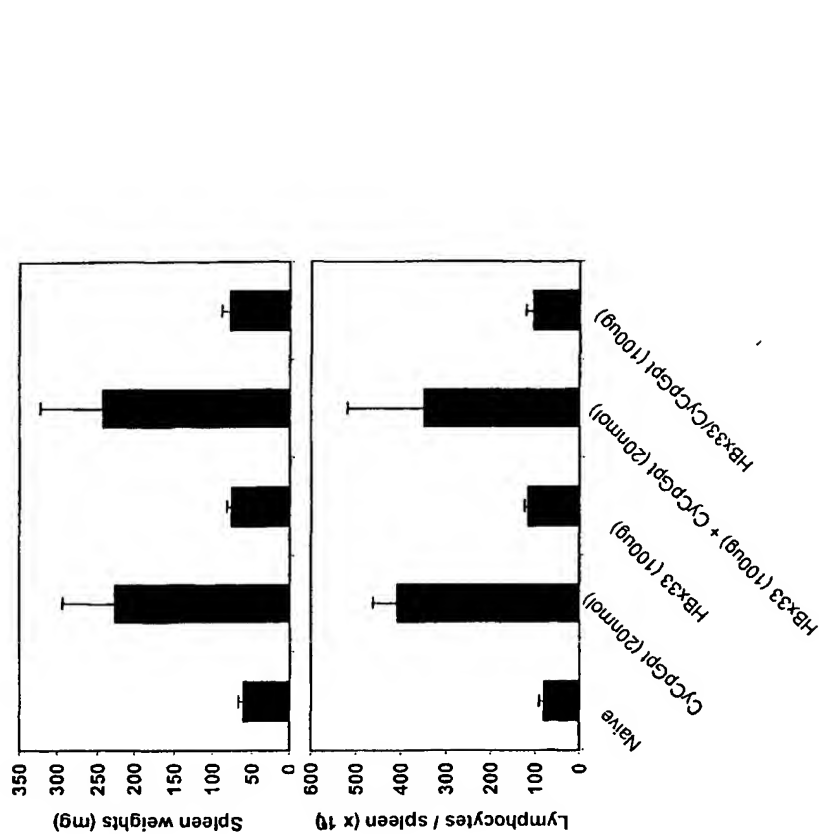


FIG. 48

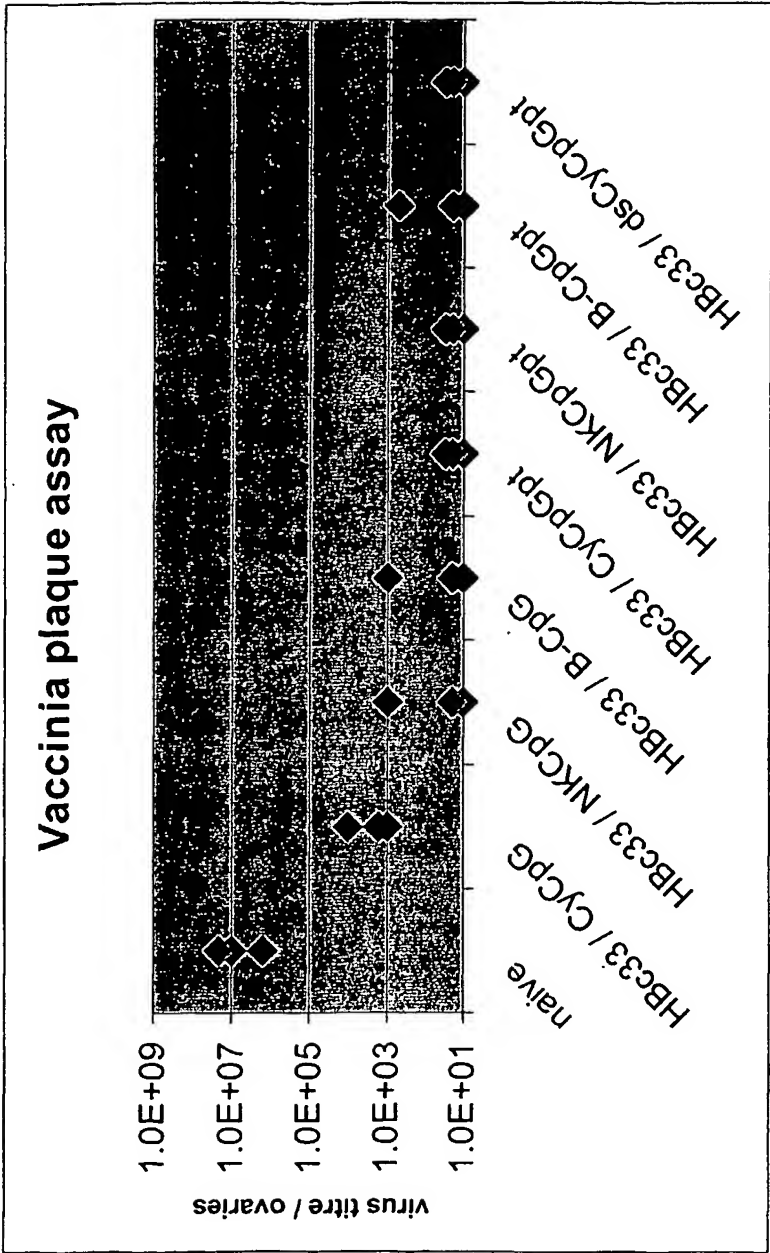


FIG. 49

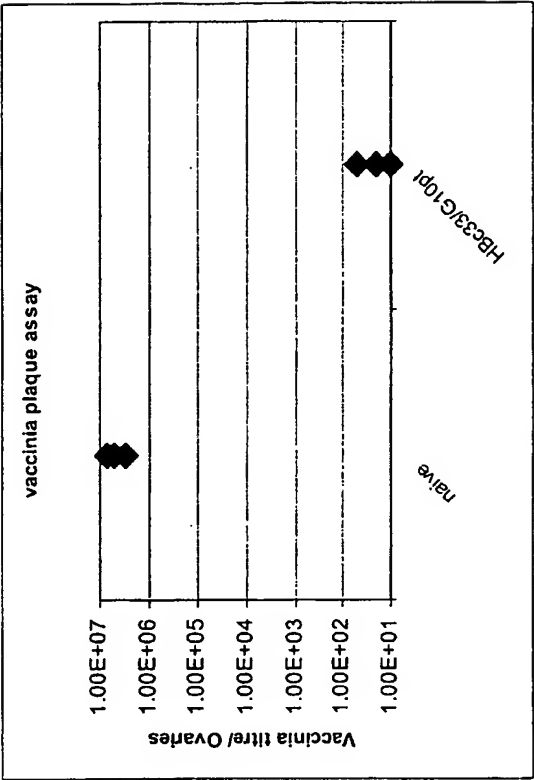


FIG. 50

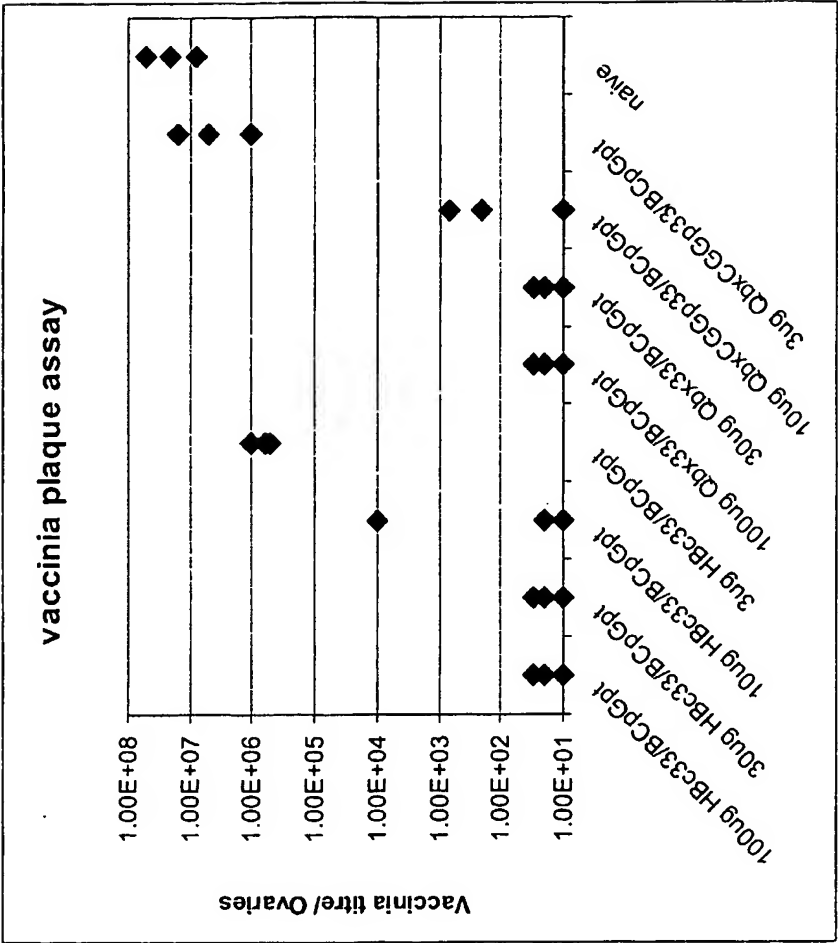


FIG. 51

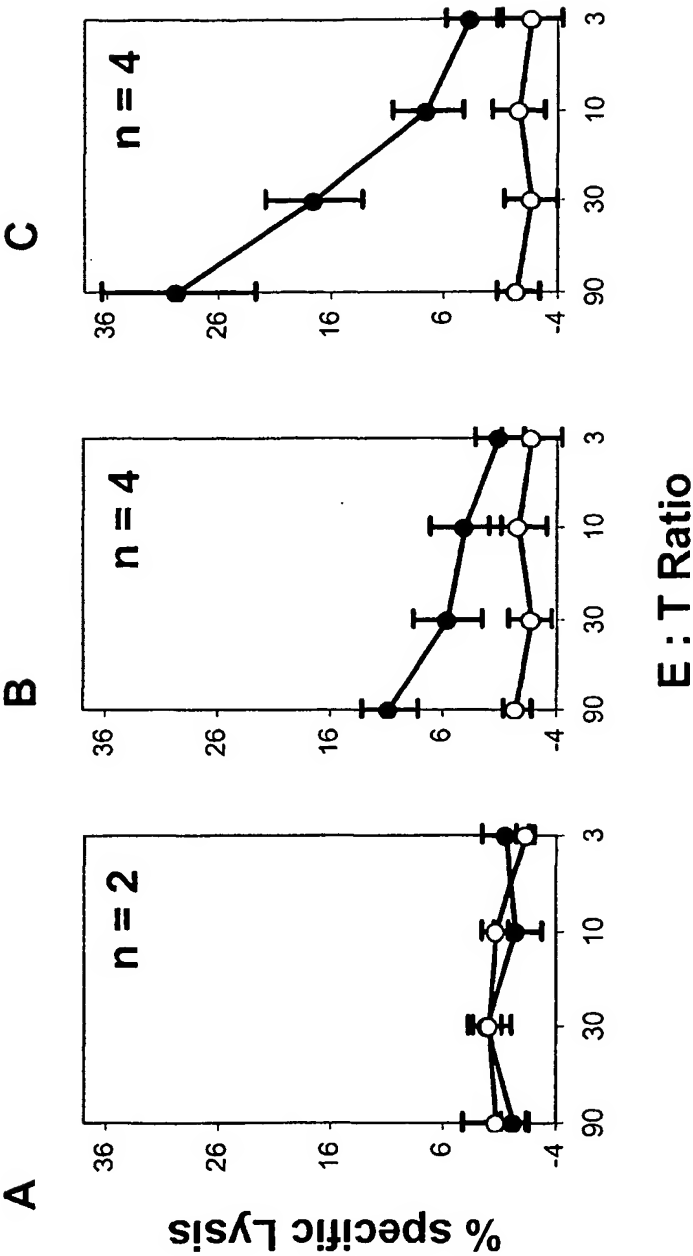


FIG. 52



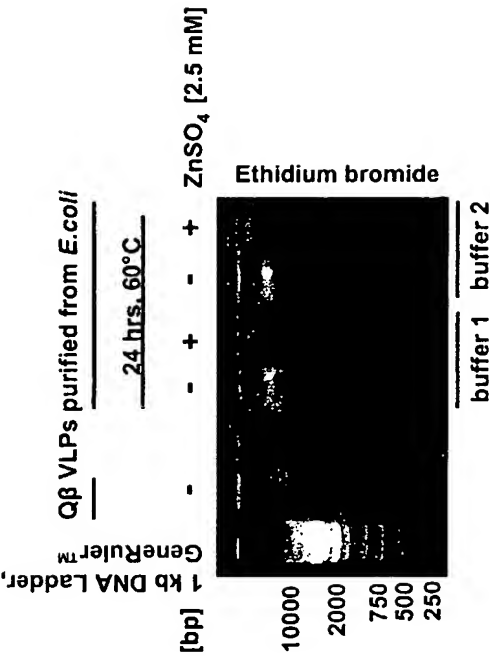


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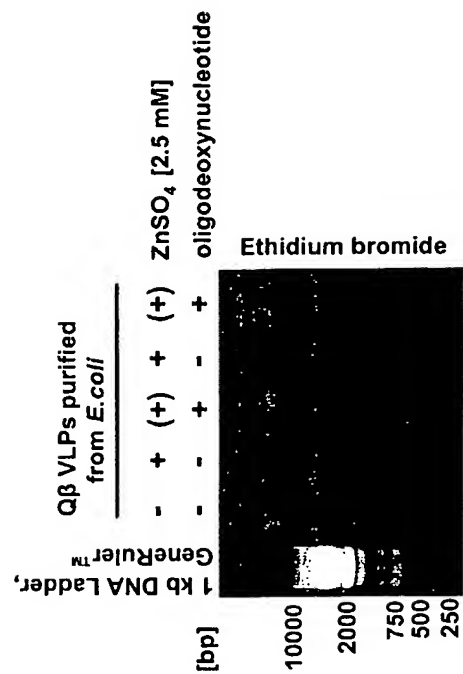


FIG. 54

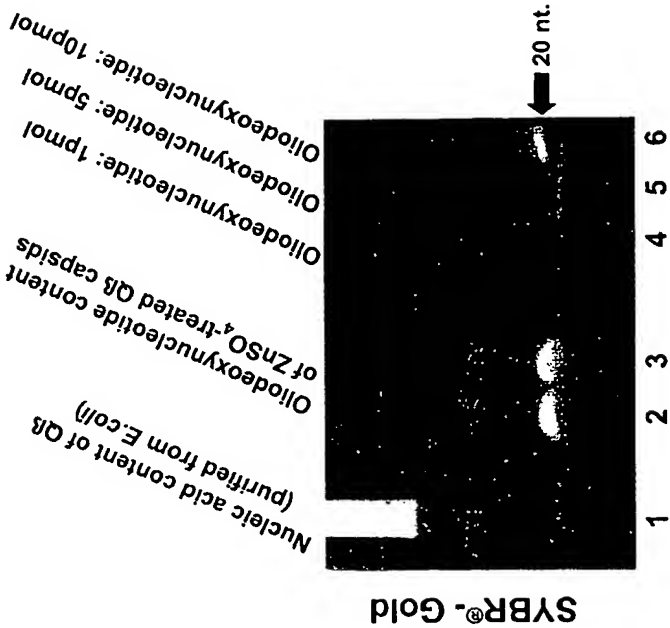


FIG. 55

-1-

## SEQUENCE LISTING

- <110> Cytos Biotechnology AG  
Bachmann, Martin F.  
Storni, Tazio  
Maurer, Patrick  
Tissot, Alain  
Schwarz, Katrin  
Meijerink, Edwin  
Lipowsky, Gerad  
Pumpens, Paul  
Cielens, Indulis  
Renhofa, Regina
- <120> Packaging of Immunostimulatory Substances into Virus-like Particles:  
Method of Preparation and Use
- <130> 1700.022PC02
- <140> To be assigned
- <141> Herewith
- <150> 60/374,145
- <151> 2002-04-22
- <150> 60/318,994
- <151> 2001-09-14
- <160> 73
- <170> PatentIn version 3.1
- <210> 10
- <211> 132
- <212> PRT

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 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
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Asn Pro Ala Tyr  
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&lt;400&gt; 11

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Lys Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly  
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-3-

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Val Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
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Val Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser  
 65 70 75 80

Cys Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser  
 85 90 95

Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu  
 100 105 110

Leu Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln  
 115 120 125

Leu Asn Pro Ala Tyr Trp Leu Leu Ile Ala Gly Gly Gly Ser Gly Ser  
 130 135 140

Lys Pro Asp Pro Val Ile Pro Asp Pro Pro Ile Asp Pro Pro Pro Gly  
 145 150 155 160

Thr Gly Lys Tyr Thr Cys Pro Phe Ala Ile Trp Ser Leu Glu Glu Val  
 165 170 175

Tyr Glu Pro Pro Thr Lys Asn Arg Pro Trp Pro Ile Tyr Asn Ala Val  
 180 185 190

Glu Leu Gln Pro Arg Glu Phe Asp Val Ala Leu Lys Asp Leu Leu Gly  
 195 200 205

Asn Thr Lys Trp Arg Asp Trp Asp Ser Arg Leu Ser Tyr Thr Thr Phe  
 210 215 220

Arg Gly Cys Arg Gly Asn Gly Tyr Ile Asp Leu Asp Ala Thr Tyr Leu  
 225 230 235 240

Ala Thr Asp Gln Ala Met Arg Asp Gln Lys Tyr Asp Ile Arg Glu Gly  
 245 250 255

Lys Lys Pro Gly Ala Phe Gly Asn Ile Glu Arg Phe Ile Tyr Leu Lys  
 260 265 270

Ser Ile Asn Ala Tyr Cys Ser Leu Ser Asp Ile Ala Ala Tyr His Ala  
 275 280 285

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Asp Gly Val Ile Val Gly Phe Trp Arg Asp Pro Ser Ser Gly Gly Ala  
 290 295 300

Ile Pro Phe Asp Phe Thr Lys Phe Asp Lys Thr Lys Cys Pro Ile Gln  
 305 310 315 320

Ala Val Ile Val Val Pro Arg Ala  
 325

<210> 12

<211> 129

<212> PRT

<213> Bacteriophage R17

<400> 12

Ala Ser Asn Phe Thr Gln Phe Val Leu Val Asn Asp Gly Gly Thr Gly  
 1 5 10 15

Asn Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu Trp  
 20 25 30

Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val  
 35 40 45

Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val  
 50 55 60

Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala  
 65 70 75 80

Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe Ala  
 85 90 95

Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu  
 100 105 110

Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile  
 115 120 125

Tyr

<210> 13

-5-

&lt;211&gt; 130

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage fr

&lt;400&gt; 13

Met Ala Ser Asn Phe Glu Glu Phe Val Leu Val Asp Asn Gly Gly Thr  
 1 5 10 15

Gly Asp Val Lys Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu  
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser  
 35 40 45

Val Arg Gln Ser Ser Ala Asn Asn Arg Lys Tyr Thr Val Lys Val Glu  
 50 55 60

Val Pro Lys Val Ala Thr Gln Val Gln Gly Gly Val Glu Leu Pro Val  
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Met Asn Met Glu Leu Thr Ile Pro Val Phe  
 85 90 95

Ala Thr Asn Asp Asp Cys Ala Leu Ile Val Lys Ala Leu Gln Gly Thr  
 100 105 110

Phe Lys Thr Gly Asn Pro Ile Ala Thr Ala Ile Ala Ala Asn Ser Gly  
 115 120 125

Ile Tyr  
 130

&lt;210&gt; 14

&lt;211&gt; 130

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage GA

&lt;400&gt; 14

Met Ala Thr Leu Arg Ser Phe Val Leu Val Asp Asn Gly Gly Thr Gly  
 1 5 10 15



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Asn Val Thr Val Val Pro Val Ser Asn Ala Asn Gly Val Ala Glu Trp  
 20 25 30

Leu Ser Asn Asn Ser Arg Ser Gln Ala Tyr Arg Val Thr Ala Ser Tyr  
 35 40 45

Arg Ala Ser Gly Ala Asp Lys Arg Lys Tyr Ala Ile Lys Leu Glu Val  
 50 55 60

Pro Lys Ile Val Thr Gln Val Val Asn Gly Val Glu Leu Pro Gly Ser  
 65 70 75 80

Ala Trp Lys Ala Tyr Ala Ser Ile Asp Leu Thr Ile Pro Ile Phe Ala  
 85 90 95

Ala Thr Asp Asp Val Thr Val Ile Ser Lys Ser Leu Ala Gly Leu Phe  
 100 105 110

Lys Val Gly Asn Pro Ile Ala Glu Ala Ile Ser Ser Gln Ser Gly Phe  
 115 120 125

Tyr Ala  
 130

<210> 15

<211> 132

<212> PRT

<213> Bacteriophage SP

<400> 15

Met Ala Lys Leu Asn Gln Val Thr Leu Ser Lys Ile Gly Lys Asn Gly  
 1 5 10 15

Asp Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly  
 20 25 30

Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
 35 40 45

Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Phe Lys  
 50 55 60

Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Arg Asp Ala Cys  
 65 70 75 80

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Asp Pro Ser Val Thr Arg Ser Ala Phe Ala Asp Val Thr Leu Ser Phe  
85 90 95

Thr Ser Tyr Ser Thr Asp Glu Glu Arg Ala Leu Ile Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Ala Asp Pro Leu Ile Val Asp Ala Ile Asp Asn Leu  
115 120 125

Asn Pro Ala Tyr  
130

<210> 16

<211> 329

<212> PRT

<213> Bacteriophage SP

<400> 16

Ala Lys Leu Asn Gln Val Thr Leu Ser Lys Ile Gly Lys Asn Gly Asp  
1 5 10 15

Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
20 25 30

Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
35 40 45

Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Phe Lys Val  
50 55 60

Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Arg Asp Ala Cys Asp  
65 70 75 80

Pro Ser Val Thr Arg Ser Ala Phe Ala Asp Val Thr Leu Ser Phe Thr  
85 90 95

Ser Tyr Ser Thr Asp Glu Glu Arg Ala Leu Ile Arg Thr Glu Leu Ala  
100 105 110

Ala Leu Leu Ala Asp Pro Leu Ile Val Asp Ala Ile Asp Asn Leu Asn  
115 120 125

Pro Ala Tyr Trp Ala Ala Leu Leu Val Ala Ser Ser Gly Gly Gly Asp

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130                      135                      140  
 Asn Pro Ser Asp Pro Asp Val Pro Val Val Pro Asp Val Lys Pro Pro  
 145                      150                      155                      160  
 Asp Gly Thr Gly Arg Tyr Lys Cys Pro Phe Ala Cys Tyr Arg Leu Gly  
                                  165                      170                      175  
 Ser Ile Tyr Glu Val Gly Lys Glu Gly Ser Pro Asp Ile Tyr Glu Arg  
                                  180                      185                      190  
 Gly Asp Glu Val Ser Val Thr Phe Asp Tyr Ala Leu Glu Asp Phe Leu  
                                  195                      200                      205  
 Gly Asn Thr Asn Trp Arg Asn Trp Asp Gln Arg Leu Ser Asp Tyr Asp  
                                  210                      215                      220  
 Ile Ala Asn Arg Arg Arg Cys Arg Gly Asn Gly Tyr Ile Asp Leu Asp  
 225                      230                      235                      240  
 Ala Thr Ala Met Gln Ser Asp Asp Phe Val Leu Ser Gly Arg Tyr Gly  
                                  245                      250                      255  
 Val Arg Lys Val Lys Phe Pro Gly Ala Phe Gly Ser Ile Lys Tyr Leu  
                                  260                      265                      270  
 Leu Asn Ile Gln Gly Asp Ala Trp Leu Asp Leu Ser Glu Val Thr Ala  
                                  275                      280                      285  
 Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser  
                                  290                      295                      300  
 Pro Gln Leu Pro Thr Asp Phe Thr Gln Phe Asn Ser Ala Asn Cys Pro  
 305                      310                      315                      320  
 Val Gln Thr Val Ile Ile Ile Pro Ser  
                                  325

&lt;210&gt; 17

&lt;211&gt; 130

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage MS2

&lt;400&gt; 17

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Met Ala Ser Asn Phe Thr Gln Phe Val Leu Val Asp Asn Gly Gly Thr  
 1 5 10 15

Gly Asp Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu  
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser  
 35 40 45

Val Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu  
 50 55 60

Val Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val  
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe  
 85 90 95

Ala Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu  
 100 105 110

Leu Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly  
 115 120 125

Ile Tyr  
 130

<210> 18

<211> 133

<212> PRT

<213> Bacteriophage M11

<400> 18

Met Ala Lys Leu Gln Ala Ile Thr Leu Ser Gly Ile Gly Lys Lys Gly  
 1 5 10 15

Asp Val Thr Leu Asp Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly  
 20 25 30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
 35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
 50 55 60

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Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr  
65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ser Asp Val Thr Phe Ser  
85 90 95

Phe Thr Gln Tyr Ser Thr Val Glu Glu Arg Ala Leu Val Arg Thr Glu  
100 105 110

Leu Gln Ala Leu Leu Ala Asp Pro Met Leu Val Asn Ala Ile Asp Asn  
115 120 125

Leu Asn Pro Ala Tyr  
130

<210> 19

<211> 133

<212> PRT

<213> Bacteriophage MX1

<400> 19

Met Ala Lys Leu Gln Ala Ile Thr Leu Ser Gly Ile Gly Lys Asn Gly  
1 5 10 15

Asp Val Thr Leu Asn Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly  
20 25 30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr  
65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ala Asp Val Thr Phe Ser  
85 90 95

Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Leu Val Arg Thr Glu  
100 105 110

Leu Lys Ala Leu Leu Ala Asp Pro Met Leu Ile Asp Ala Ile Asp Asn

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115

120

125

Leu Asn Pro Ala Tyr  
130

&lt;210&gt; 20

&lt;211&gt; 330

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage NL95

&lt;400&gt; 20

Met Ala Lys Leu Asn Lys Val Thr Leu Thr Gly Ile Gly Lys Ala Gly  
1 5 10 15

Asn Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly  
20 25 30

Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
35 40 45

Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
50 55 60

Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Lys Asp Ala Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Ser Gly Ser Arg Asp Val Thr Leu Ser Phe  
85 90 95

Thr Ser Tyr Ser Thr Glu Arg Glu Arg Ala Leu Ile Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Lys Asp Asp Leu Ile Val Asp Ala Ile Asp Asn Leu  
115 120 125

Asn Pro Ala Tyr Trp Ala Ala Leu Leu Ala Ala Ser Pro Gly Gly Gly  
130 135 140

Asn Asn Pro Tyr Pro Gly Val Pro Asp Ser Pro Asn Val Lys Pro Pro  
145 150 155 160

Gly Gly Thr Gly Thr Tyr Arg Cys Pro Phe Ala Cys Tyr Arg Arg Gly  
165 170 175

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Glu Leu Ile Thr Glu Ala Lys Asp Gly Ala Cys Ala Leu Tyr Ala Cys  
                   180                  185                  190

Gly Ser Glu Ala Leu Val Glu Phe Glu Tyr Ala Leu Glu Asp Phe Leu  
                   195                  200                  205

Gly Asn Glu Phe Trp Arg Asn Trp Asp Gly Arg Leu Ser Lys Tyr Asp  
                   210                  215                  220

Ile Glu Thr His Arg Arg Cys Arg Gly Asn Gly Tyr Val Asp Leu Asp  
                   225                  230                  235                  240

Ala Ser Val Met Gln Ser Asp Glu Tyr Val Leu Ser Gly Ala Tyr Asp  
                   245                  250                  255

Val Val Lys Met Gln Pro Pro Gly Thr Phe Asp Ser Pro Arg Tyr Tyr  
                   260                  265                  270

Leu His Leu Met Asp Gly Ile Tyr Val Asp Leu Ala Glu Val Thr Ala  
                   275                  280                  285

Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser  
                   290                  295                  300

Pro Gln Leu Pro Thr Asp Phe Thr Arg Phe Asn Arg His Asn Cys Pro  
                   305                  310                  315                  320

Val Gln Thr Val Ile Val Ile Pro Ser Leu  
                   325                  330

<210> 21

<211> 129

<212> PRT

<213> Bacteriophage f2

<400> 21

Ala Ser Asn Phe Thr Gln Phe Val Leu Val Asn Asp Gly Gly Thr Gly  
                   1                  5                  10                  15

Asn Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu Trp  
                   20                  25                  30

Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val  
                   35                  40                  45

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Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val  
50 55 60

Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala  
65 70 75 80

Ala Trp Arg Ser Tyr Leu Asn Leu Glu Leu Thr Ile Pro Ile Phe Ala  
85 90 95

Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu  
100 105 110

Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile  
115 120 125

Tyr

<210> 22

<211> 128

<212> PRT

<213> Bacteriophage PP7

<400> 22

Met Ser Lys Thr Ile Val Leu Ser Val Gly Glu Ala Thr Arg Thr Leu  
1 5 10 15

Thr Glu Ile Gln Ser Thr Ala Asp Arg Gln Ile Phe Glu Glu Lys Val  
20 25 30

Gly Pro Leu Val Gly Arg Leu Arg Leu Thr Ala Ser Leu Arg Gln Asn  
35 40 45

Gly Ala Lys Thr Ala Tyr Arg Val Asn Leu Lys Leu Asp Gln Ala Asp  
50 55 60

Val Val Asp Cys Ser Thr Ser Val Cys Gly Glu Leu Pro Lys Val Arg  
65 70 75 80

Tyr Thr Gln Val Trp Ser His Asp Val Thr Ile Val Ala Asn Ser Thr  
85 90 95

Glu Ala Ser Arg Lys Ser Leu Tyr Asp Leu Thr Lys Ser Leu Val Ala



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100 105 110  
 Thr Ser Gln Val Glu Asp Leu Val Val Asn Leu Val Pro Leu Gly Arg  
 115 120 125  
  
 <210> 23  
 <211> 132  
 <212> PRT  
 <213> Bacteriophage Q-beta  
  
 <400> 23  
 Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Arg Asp Gly Lys  
 1 5 10 15  
 Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30  
 Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45  
 Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60  
 Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80  
 Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95  
 Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110  
 Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125  
 Asn Pro Ala Tyr  
 130

<210> 24  
 <211> 132  
 <212> PRT  
 <213> Bacteriophage Q-beta

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&lt;400&gt; 24

Ala Lys Leu Glu Thr Val Thr Leu Gly Lys Ile Gly Lys Asp Gly Lys  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

&lt;210&gt; 25

&lt;211&gt; 132

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage Q-beta

&lt;400&gt; 25

Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Arg Asp Gly Lys  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

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Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

<210> 26

<211> 132

<212> PRT

<213> Bacteriophage Q-beta

<400> 26

Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu

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100

105

110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

&lt;210&gt; 27

&lt;211&gt; 132

&lt;212&gt; .PRT

&lt;213&gt; Bacteriophage Q-beta

&lt;400&gt; 27

Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

&lt;210&gt; 28

&lt;211&gt; 184

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&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 28

Met Asp Ile Asp Pro Tyr Glu Phe Gly Ala Thr Val Glu Leu Leu Ser  
 1 5 10 15

Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr  
 20 25 30

Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser  
 35 40 45

Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu  
 50 55 60

Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala Ser  
 65 70 75 80

Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys Ile  
 85 90 95

Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu  
 100 105 110

Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro  
 115 120 125

Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu  
 130 135 140

Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg Arg  
 145 150 155 160

Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg  
 165 170 175

Ser Gln Ser Arg Glu Ser Gln Cys  
 180

&lt;210&gt; 29

&lt;211&gt; 183

&lt;212&gt; PRT

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&lt;213&gt; Hepatitis B virus

&lt;400&gt; 29

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Gly Ser Gln Cys  
 180

&lt;210&gt; 30

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

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&lt;400&gt; 30

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Thr Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Cys Val Ile Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Gly Ser Gln Cys  
 180

&lt;210&gt; 31

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 31

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Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 32

<211> 212

<212> PRT



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&lt;213&gt; Hepatitis B virus

&lt;400&gt; 32

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Asn Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

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&lt;210&gt; 33

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 33

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Thr Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Glu Ser Gln Cys  
 180

&lt;210&gt; 34

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&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 34

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

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Glu Ser Gln Cys  
210

<210> 35

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 35

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

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Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 36

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 36

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro Gln  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr

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	165		170		175
Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro	180	185	190		
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg	195	200	205		
Glu Ser Gln Cys	210				
<210> 37					
<211> 212					
<212> PRT					
<213> Hepatitis B virus					
<400> 37					
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr	1	5	10	15	
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile	20	25	30		
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu	35	40	45		
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser	50	55	60		
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His	65	70	75	80	
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr	85	90	95		
Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp	100	105	110		
Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln	115	120	125		
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val	130	135	140		

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Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Lys Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Gly Ser Gln Cys  
210

<210> 38

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 38

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Phe Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ala  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Asp Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

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Pro Pro Ala Tyr Arg Pro Ser Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Glu Ser Gln Cys  
 180

<210> 39

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 39

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro



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130                      135                      140  
 Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145                      150                      155                      160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165                      170                      175  
 Gln Ser Arg Glu Ser Gln Cys  
 180  
 <210> 40  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus  
 <400> 40  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1                      5                      10                      15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20                      25                      30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35                      40                      45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50                      55                      60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65                      70                      75                      80  
 His Thr Ala Leu Arg His Ala Ile Leu Cys Trp Gly Asp Leu Arg Thr  
 85                      90                      95  
 Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100                      105                      110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115                      120                      125  
 Leu Leu Tyr Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130                      135                      140

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Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 41

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 41

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Phe Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Ala Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

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Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Gln Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Cys  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 42

<211> 183

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic human Hepatitis B virus core protein gene

<400> 42

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys

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			85						90						95
Phe	Arg	Gln	Leu	Leu	Trp	Phe	His	Ile	Ser	Cys	Leu	Thr	Phe	Gly	Arg
			100					105					110		
Glu	Thr	Val	Leu	Glu	Tyr	Leu	Val	Ser	Phe	Gly	Val	Trp	Ile	Arg	Thr
		115					120					125			
Pro	Pro	Ala	Tyr	Arg	Pro	Pro	Asn	Ala	Pro	Ile	Leu	Ser	Thr	Leu	Pro
		130					135				140				
Glu	Thr	Thr	Val	Val	Arg	Arg	Arg	Gly	Arg	Ser	Pro	Arg	Arg	Arg	Thr
145					150					155					160
Pro	Ser	Pro	Arg	Arg	Arg	Arg	Ser	Gln	Ser	Pro	Arg	Arg	Arg	Arg	Ser
				165					170						175
Gln	Ser	Arg	Glu	Ser	Gln	Cys									
			180												

&lt;210&gt; 43

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 43

Met	Gln	Leu	Phe	His	Leu	Cys	Leu	Ile	Ile	Ser	Cys	Ser	Cys	Pro	Thr
1				5					10					15	
Val	Gln	Ala	Ser	Lys	Leu	Cys	Leu	Gly	Trp	Leu	Trp	Gly	Met	Asp	Ile
			20					25					30		
Asp	Pro	Tyr	Lys	Glu	Phe	Gly	Ala	Thr	Val	Glu	Leu	Leu	Ser	Phe	Leu
		35					40					45			
Pro	Ser	Asp	Phe	Phe	Pro	Ser	Val	Arg	Asp	Leu	Leu	Asp	Thr	Ala	Ser
	50					55					60				
Ala	Leu	Tyr	Arg	Glu	Ala	Leu	Glu	Ser	Pro	Glu	His	Cys	Ser	Pro	His
65					70					75					80
His	Thr	Ala	Leu	Arg	Gln	Ala	Ile	Leu	Cys	Trp	Gly	Asp	Leu	Met	Ser
			85					90						95	

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Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 44

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 44

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
 65 70 75 80

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Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

Gln Ser Arg Glu Ser Gln Cys  
180

&lt;210&gt; 45

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 45

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys

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85                      90                      95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                     100                      105                      110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                     115                      120                      125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                     130                      135                      140  
 Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
                     145                      150                      155                      160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                     165                      170                      175  
 Gln Ser Arg Glu Ser Gln Cys  
                     180

&lt;210&gt; 46

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 46

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1                      5                      10                      15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                     20                      25                      30  
 Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
                     35                      40                      45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
                     50                      55                      60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Ala Asn Leu Glu Asp Pro Ala  
 65                      70                      75                      80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                     85                      90                      95

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Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                   100                  105                  110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
           115                  120                  125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
       130                  135                  140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Thr Pro Arg Arg Arg Thr  
   145                  150                  155                  160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                   165                  170                  175

Gln Ser Arg Glu Ser Gln Cys  
           180

<210> 47

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 47

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                  5                  10                  15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
           20                  25                  30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
       35                  40                  45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
       50                  55                  60

Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
   65                  70                  75                  80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
           85                  90                  95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
       100                  105                  110



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Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 48

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 48

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr

85

90

95

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 49

<211>    212

<212> PRT

<213> Hepatitis B virus

<400> 49

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Thr Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Gln Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

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Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ala Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 50

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 50

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

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Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Phe Glu Cys Ser Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 51

<211> 212

<212> PRT

<213> Hepatitis B virus

<220>

<221> MISC\_FEATURE

<222> (28)..(28)

<223> Xaa can be any amino acid

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&lt;400&gt; 51

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Xaa Asp Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Ile Thr  
 85 90 95

Leu Ser Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Thr Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 52

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&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 52

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Asn Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

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Glu Ser Gln Cys  
210

<210> 53

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 53 .

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Cys Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

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Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 54

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 54

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr



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165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Pro Gln Cys  
210

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<210> 55
<211> 212
<212> PRT
<213> Hepatitis B virus
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<400> 55
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
1          5          10          15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
          20          25          30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
          35          40          45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Ser Thr Ala Ser
          50          55          60
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
65          70          75          80
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr
          85          90          95
Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp
          100          105          110
Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln
          115          120          125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
          130          135          140

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Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 56

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 56

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

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Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Leu Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 57

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 57

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp

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100	105	110
Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Lys Gln		
115	120	125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val		
130	135	140
Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala		
145	150	155
Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr		
	165	170
Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro		
	180	185
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg		
	195	200
Glu Ser Gln Cys		
210		

&lt;210&gt; 58

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 58

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
1 5 10 15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
20 25 30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
35 40 45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ala
50 55 60
Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
65 70 75 80

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His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Sér Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 59

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 59

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Ser Met Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Tyr Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Thr Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

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Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Gln Asp Pro Thr  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Val Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Val Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Gln Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

Gln Ser Arg Glu Ser Gln Cys  
180

<210> 60

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 60

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg His Val Phe Leu Cys Trp Gly Asp  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr

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[illegible]

<210> 61

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 61

Met	Gln	Leu	Phe	His	Leu	Cys	Leu	Ile	Ile	Ser	Cys	Ser	Cys	Pro	Thr
1				5					10					15	
Val	Gln	Ala	Ser	Lys	Leu	Cys	Leu	Gly	Trp	Leu	Trp	Gly	Met	Asp	Ile
			20					25					30		
Asp	Pro	Tyr	Lys	Glu	Phe	Gly	Ala	Thr	Val	Glu	Leu	Leu	Ser	Phe	Leu
		35					40					45			
Pro	Ser	Asp	Phe	Phe	Pro	Ser	Val	Arg	Asp	Leu	Leu	Asp	Thr	Ala	Ser
	50					55					60				
Ala	Leu	Tyr	Arg	Glu	Ala	Leu	Glu	Ser	Pro	Glu	His	Cys	Ser	Pro	His
65					70					75					80

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His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Thr Thr  
85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 62

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 62

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60



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Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Ile Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 63

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 63

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys

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35                      40                      45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
   50                      55                      60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Val  
   65                      70                      75                      80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys  
                   85                      90                      95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                  100                      105                      110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                  115                      120                      125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                  130                      135                      140  
 Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
   145                      150                      155                      160  
 Pro Ser Pro Ala Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                  165                      170                      175  
 Gln Ser Arg Glu Ser Gln Cys  
                  180

&lt;210&gt; 64

&lt;211&gt; 213

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 64

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                      5                      10                      15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                  20                      25                      30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                  35                      40                      45

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Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Asn  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp  
 100 105 110

Leu Val Val Gly Tyr Val Asn Thr Thr Val Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser  
 195 200 205

Arg Glu Ser Gln Cys  
 210

<210> 65

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 65

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

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Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
           35                                  40                                  45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
           50                                  55                                  60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
   65                                  70                                  75                                  80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                                   85                                  90                                  95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                                   100                                  105                                  110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
           115                                  120                                  125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
   130                                  135                                  140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Thr Pro Arg Arg Arg Thr  
   145                                  150                                  155                                  160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                                   165                                  170                                  175

Gln Ser Arg Glu Ser Gln Cys  
                                   180

<210> 66

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 66

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                                  5                                  10                                  15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
           20                                  25                                  30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu

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35

40

45

Pro Ser Asp Phe Phe Pro Ser Val Arg Ala Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Ile Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 67

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 67

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

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Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Thr Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

&lt;210&gt; 68

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

-60-

&lt;400&gt; 68

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Arg Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Thr Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 69

&lt;211&gt; 212

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&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 69

Met Gln Leu Phe His Leu Cys Leu Val Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ala  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys Ile Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210



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&lt;210&gt; 70

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 70

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Ala Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg

-63-

195

200

205

Glu Ser Gln Cys  
210

&lt;210&gt; 71

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 71

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly/Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ala Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

-64-

Gln Ser Arg Glu Ser Gln Cys  
180

<210> 72

<211> 183

<212> PRT

<213> Hepatitis B virus

<400> 72

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

Gln Ser Arg Gly Ser Gln Cys  
180

-65-

&lt;210&gt; 73

&lt;211&gt; 188

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 73

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu  
 1 5 10 15

Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp  
 20 25 30

Thr Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu  
 50 55 60

Leu Thr Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln  
 65 70 75 80

Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys  
 85 90 95

Val Arg Gln Ser Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln  
 100 105 110

His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Ala Pro Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu His Thr Val Ile Arg Arg Arg Gly Gly Ala Arg Ala Ser Arg Ser  
 145 150 155 160

Pro Arg Arg Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro  
 165 170 175

Arg Arg Arg Arg Ser Gln Ser Pro Ser Thr Asn Cys  
 180 185

\  
 <210> 74

-66-

&lt;211&gt; 217

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 74

Met Tyr Leu Phe His Leu Cys Leu Val Phe Ala Cys Val Pro Cys Pro  
 1 5 10 15

Thr Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp  
 20 25 30

Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu Asn Phe  
 35 40 45

Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp Thr Ala  
 50 55 60

Ala Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys Ser Pro  
 65 70 75 80

His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Glu Glu Leu Thr  
 85 90 95

Arg Leu Ile Thr Trp Met Ser Glu Asn Thr Thr Glu Glu Val Arg Arg  
 100 105 110

Ile Ile Val Asp His Val Asn Asn Thr Trp Gly Leu Lys Val Arg Gln  
 115 120 125

Thr Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln His Thr Val  
 130 135 140

Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Ala Pro  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu His Thr  
 165 170 175

Val Ile Arg Arg Arg Gly Gly Ser Arg Ala Ala Arg Ser Pro Arg Arg  
 180 185 190

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg  
 195 200 205

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Arg Ser Gln Ser Pro Ala Ser Asn Cys  
 210 215

<210> 75

<211> 262

<212> PRT

<213> Hepatitis B virus

<400> 75

Met Asp Val Asn Ala Ser Arg Ala Leu Ala Asn Val Tyr Asp Leu Pro  
 1 5 10 15

Asp Asp Phe Phe Pro Lys Ile Glu Asp Leu Val Arg Asp Ala Lys Asp  
 20 25 30

Ala Leu Glu Pro Tyr Trp Lys Ser Asp Ser Ile Lys Lys His Val Leu  
 35 40 45

Ile Ala Thr His Phe Val Asp Leu Ile Glu Asp Phe Trp Gln Thr Thr  
 50 55 60

Gln Gly Met His Glu Ile Ala Glu Ala Ile Arg Ala Val Ile Pro Pro  
 65 70 75 80

Thr Thr Ala Pro Val Pro Ser Gly Tyr Leu Ile Gln His Asp Glu Ala  
 85 90 95

Glu Glu Ile Pro Leu Gly Asp Leu Phe Lys Glu Gln Glu Glu Arg Ile  
 100 105 110

Val Ser Phe Gln Pro Asp Tyr Pro Ile Thr Ala Arg Ile His Ala His  
 115 120 125

Leu Lys Ala Tyr Ala Lys Ile Asn Glu Glu Ser Leu Asp Arg Ala Arg  
 130 135 140

Arg Leu Leu Trp Trp His Tyr Asn Cys Leu Leu Trp Gly Glu Ala Thr  
 145 150 155 160

Val Thr Asn Tyr Ile Ser Arg Leu Arg Thr Trp Leu Ser Thr Pro Glu  
 165 170 175

Lys Tyr Arg Gly Arg Asp Ala Pro Thr Ile Glu Ala Ile Thr Arg Pro  
 180 185 190

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Ile Gln Val Ala Gln Gly Gly Arg Lys Thr Ser Thr Ala Thr Arg Lys  
 195 200 205

Pro Arg Gly Leu Glu Pro Arg Arg Arg Lys Val Lys Thr Thr Val Val  
 210 215 220

Tyr Gly Arg Arg Arg Ser Lys Ser Arg Glu Arg Arg Ala Ser Ser Pro  
 225 230 235 240

Gln Arg Ala Gly Ser Pro Leu Pro Arg Ser Ser Ser Ser His His Arg  
 245 250 255

Ser Pro Ser Pro Arg Lys  
 260

<210> 76

<211> 305

<212> PRT

<213> Hepatitis B virus

<400> 76

Met Trp Asp Leu Arg Leu His Pro Ser Pro Phe Gly Ala Ala Cys Gln  
 1 5 10 15

Gly Ile Phe Thr Ser Ser Leu Leu Leu Phe Leu Val Thr Val Pro Leu  
 20 25 30

Val Cys Thr Ile Val Tyr Asp Ser Cys Leu Cys Met Asp Ile Asn Ala  
 35 40 45

Ser Arg Ala Leu Ala Asn Val Tyr Asp Leu Pro Asp Asp Phe Phe Pro  
 50 55 60

Lys Ile Asp Asp Leu Val Arg Asp Ala Lys Asp Ala Leu Glu Pro Tyr  
 65 70 75 80

Trp Arg Asn Asp Ser Ile Lys Lys His Val Leu Ile Ala Thr His Phe  
 85 90 95

Val Asp Leu Ile Glu Asp Phe Trp Gln Thr Thr Gln Gly Met His Glu  
 100 105 110

Ile Ala Glu Ala Leu Arg Ala Ile Ile Pro Ala Thr Thr Ala Pro Val

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115                      120                      125  
 Pro Gln Gly Phe Leu Val Gln His Glu Glu Ala Glu Glu Ile Pro Leu  
     130                      135                      140  
 Gly Glu Leu Phe Arg Tyr Gln Glu Glu Arg Leu Thr Asn Phe Gln Pro  
     145                      150                      155                      160  
 Asp Tyr Pro Val Thr Ala Arg Ile His Ala His Leu Lys Ala Tyr Ala  
                     165                      170                      175  
 Lys Ile Asn Glu Glu Ser Leu Asp Arg Ala Arg Arg Leu Leu Trp Trp  
                     180                      185                      190  
 His Tyr Asn Cys Leu Leu Trp Gly Glu Pro Asn Val Thr Asn Tyr Ile  
                     195                      200                      205  
 Ser Arg Leu Arg Thr Trp Leu Ser Thr Pro Glu Lys Tyr Arg Gly Lys  
     210                      215                      220  
 Asp Ala Pro Thr Ile Glu Ala Ile Thr Arg Pro Ile Gln Val Ala Gln  
     225                      230                      235                      240  
 Gly Gly Arg Asn Lys Thr Gln Gly Val Arg Lys Ser Arg Gly Leu Glu  
                     245                      250                      255  
 Pro Arg Arg Arg Arg Val Lys Thr Thr Ile Val Tyr Gly Arg Arg Arg  
                     260                      265                      270  
 Ser Lys Ser Arg Glu Arg Arg Ala Pro Thr Pro Gln Arg Ala Gly Ser  
     275                      280                      285  
 Pro Leu Pro Arg Thr Ser Arg Asp His His Arg Ser Pro Ser Pro Arg  
     290                      295                      300

Glu  
305

&lt;210&gt; 77

&lt;211&gt; 185

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 77



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Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg  
 145 150 155 160

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg  
 165 170 175

Arg Ser Gln Ser Arg Glu Ser Gln Cys  
 180 185

<210> 78

<211> 152

<212> PRT

<213> Hepatitis B virus

<400> 78

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

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Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                   20                  25                  30  
 Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
                   35                  40                  45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
           50                  55                  60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Gly Gly  
   65                  70                  75                  80  
 Lys Gly Gly Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val  
                   85                  90                  95  
 Gly Leu Lys Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr  
                   100                  105                  110  
 Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp  
           115                  120                  125  
 Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser  
           130                  135                  140  
 Thr Leu Pro Glu Thr Thr Val Val  
   145                  150

&lt;210&gt; 79

&lt;211&gt; 3635

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; plasmid pAP283-58

&lt;400&gt; 79

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taaagttggt atagccgaac tgaataatgt ttcaggtcaa tatgtatctg ttataagcg	300

-72-

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-73-

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&lt;210&gt; 80

&lt;211&gt; 131

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;223&gt; AP205 coat protein

&lt;400&gt; 80

Met Ala Asn Lys Pro Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile  
1 5 10 15

Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu  
20 25 30

Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser  
35 40 45

Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly  
50 55 60

Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg  
65 70 75 80

Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu  
85 90 95

Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn  
100 105 110

Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp  
115 120 125

Thr Thr Ala  
130

&lt;210&gt; 81

&lt;211&gt; 131

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; AP205 coat protein

&lt;400&gt; 81

Met Ala Asn Lys Thr Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile  
1 5 10 15

Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu  
20 25 30

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Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser  
           35                                  40                                  45

Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly  
           50                                  55                                  60

Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg  
   65                                  70                                  75                                  80

Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu  
                                   85                                  90                                  95

Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn  
                                   100                                  105                                  110

Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp  
           115                                  120                                  125

Thr Thr Ala  
       130

<210> 82

<211> 3607

<212> DNA

<213> Artificial Sequence

<220>

<223> plasmid pAP281-32

<400> 82

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cggaatt	3607



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> ORDERED MOLECULAR PRESENTATION OF ANTIGENS, METHOD OF PREPARATION AND USE  <b>(57) Abstract</b>  The invention provides compositions and processes for the production of ordered and repetitive antigen or antigenic determinant arrays. The compositions of the invention are useful for the production of vaccines for the prevention of infectious diseases, the treatment of allergies and the treatment of cancers. Various embodiments of the invention provide for a virus, virus-like particle, viral capsid particle, phage or recombinant form thereof coated with any desired antigen in a highly ordered and repetitive fashion as the result of specific interactions. In one specific embodiment, a versatile new technology based on a cassette-type system (Alpha Vaccine Technology) allows production of antigen coated viral particles. Other specific embodiments allow the production of antigen coated hepatitis B virus-like particles or antigen coated Measles virus-like particles.		

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## Ordered Molecular Presentation of Antigens, Method of Preparation and Use

### *Background of the Invention*

#### *Field of the Invention*

5           The present invention is related to the fields of molecular biology, virology, immunology and medicine. The invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array. The invention also provides a process for producing an antigen or antigenic determinant in an ordered and repetitive array. The ordered and repetitive antigen  
10 or antigenic determinant is useful in the production of vaccines for the treatment of infectious diseases, the treatment of allergies and as a pharmaccine to prevent or cure cancer and to generate defined self-specific antibodies.

#### *Related Art*

15           Vaccine development for the prevention of infectious disease has had the greatest impact on human health of any medical invention. It is estimated that three million deaths per year are prevented worldwide by vaccination (Hillemann, *Nature Medicine* 4:507 (1998)). The most common vaccination strategy, the use of attenuated (*i.e.* less virulent) pathogens or closely related organisms, was first demonstrated by Edward Jenner in 1796, who vaccinated against smallpox by the  
20 administration of a less dangerous cowpox virus. Although a number of live attenuated viruses (*e. g.* measles, mumps, rubella, varicella, adenovirus, polio, influenza) and bacteria (*e. g.* bacille Calmette-Guerin (BCG) against tuberculosis) are successfully administered for vaccination, there is a risk for the development of serious complications related to a reversion to virulence and infection by the  
25 'vaccine' organism, in particular in immunocompromised individuals.

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The specific design of attenuated viruses is now enabled by recombinant DNA technology (*i.e.*, genetic engineering) through the generation of deletion or mutation variants. For example, the administration of an engineered Simian Immunodeficiency Virus (SIV) with a deletion within the *nef* gene was shown to protect macaques from subsequent infection with a pathogenic SIV strain (Daniel *et al.*, *Science* 258:1938-1941 (1992)). However, the progression of acquired immunodeficiency syndrome (AIDS)-like symptoms in animals administered attenuated SIV raises safety concerns (Baba *et al.*, *Science* 267:1820-1825 ((1995)).

As an alternative approach, attenuated viruses or bacteria may be used as carriers for the antigen-encoding genes of a pathogen that is considered too unsafe to be administered in an attenuated form (*e.g.*, Human Immunodeficiency Virus (HIV)). Upon delivery of the antigen-encoding gene to the host, the antigen is synthesized *in situ*. Vaccinia and related avipox viruses have been used as such carriers for various genes in preclinical and clinical studies for a variety of diseases (*e.g.*, Shen *et al.*, *Science* 252:440 (1991)). One disadvantage of this vaccination strategy is that it does not mimic the virion surface, because the recombinant protein is expressed on the surface of the host cell. Additionally, complications may develop in immunocompromised individuals, as evidenced by life-threatening disseminated vaccinia infections (Redfield, *N. Eng. J. Med.* 316:673 ((1998)).

A fourth vaccination approach involves the use of isolated components of a pathogen, either purified from the pathogen grown *in vitro* (*e.g.* influenza hemagglutinin or neuraminidase) or after heterologous expression of a single viral protein (*e.g.* hepatitis B surface antigen). For example, recombinant, mutated toxins (detoxified) are used for vaccination against diphtheria, tetanus, cholera and pertussis toxins (Levine *et al.*, *New generation vaccines*, 2nd edn., Marcel Dekker, Inc., New York 1997), and recombinant proteins of HIV (gp120 and full-length gp160) were evaluated as a means to induce neutralizing antibodies against HIV with disappointing results (Connor *et al.*, *J. Virol.* 72:1552 (1998)).

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Recently, promising results were obtained with soluble oligomeric gp160, that can induce CTL response and elicit antibodies with neutralizing activity against HIV-1 isolates (Van Cortt *et al.*, *J. Virol.* 71:4319 (1997)). In addition, peptide vaccines may be used in which known B- or T-cell epitopes of an antigen are coupled to a carrier molecule designed to increase the immunogenicity of the epitope by stimulating T-cell help. However, one significant problem with this approach is that it provides a limited immune response to the protein as a whole. Moreover, vaccines have to be individually designed for different MHC haplotypes. The most serious concern for this type of vaccine is that protective antiviral antibodies recognize complex, three-dimensional structures that cannot be mimicked by peptides.

A more novel vaccination strategy is the use of DNA vaccines (Donnelly *et al.*, *Ann. Rev. Immunol.* 15:617 (1997)), which may generate MHC Class I-restricted CTL responses (without the use of a live vector). This may provide broader protection against different strains of a virus by targeting epitopes from conserved internal proteins pertinent to many strains of the same virus. Since the antigen is produced with mammalian post-translational modification, conformation and oligomerization, it is more likely to be similar or identical to the wild-type protein produced by viral infection than recombinant or chemically modified proteins. However, this distinction may turn out to be a disadvantage for the application of bacterial antigens, since non-native post-translational modification may result in reduced immunogenicity. In addition, viral surface proteins are not highly organized in the absence of matrix proteins.

In addition to applications for the prevention of infectious disease, vaccine technology is now being utilized to address immune problems associated with allergies. In allergic individuals, antibodies of the IgE isotype are produced in an inappropriate humoral immune response towards particular antigens (allergens). The treatment of allergies by allergy immunotherapy requires weekly administration of successively increasing doses of the particular allergen over a period of up to 3-5 years. Presumably, 'blocking' IgG antibodies are generated

that intercept allergens in nasal or respiratory secretions or in membranes before they react with IgE antibodies on mast cells. However, no constant relationship exists between IgG titers and symptom relief. Presently, this is an extremely time- and cost-consuming process, to be considered only for patients with severe symptoms over an extended period each year.

It is well established that the administration of purified proteins alone is usually not sufficient to elicit a strong immune response; isolated antigen generally must be given together with helper substances called adjuvants. Within these adjuvants, the administered antigen is protected against rapid degradation, and the adjuvant provides an extended release of a low level of antigen.

Unlike isolated proteins, viruses induce prompt and efficient immune responses in the absence of any adjuvants both with and without T-cell help (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Although viruses often consist of few proteins, they are able to trigger much stronger immune responses than their isolated components. For B cell responses, it is known that one crucial factor for the immunogenicity of viruses is the repetitiveness and order of surface epitopes. Many viruses exhibit a quasi-crystalline surface that displays a regular array of epitopes which efficiently crosslinks epitope-specific immunoglobulins on B cells (Bachmann & Zinkernagel, *Immunol. Today* 17:553-558 (1996)). This crosslinking of surface immunoglobulins on B cells is a strong activation signal that directly induces cell-cycle progression and the production of IgM antibodies. Further, such triggered B cells are able to activate T helper cells, which in turn induce a switch from IgM to IgG antibody production in B cells and the generation of long-lived B cell memory - the goal of any vaccination (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Viral structure is even linked to the generation of anti-antibodies in autoimmune disease and as a part of the natural response to pathogens (see Fehr, T., *et al.*, *J. Exp. Med.* 185:1785-1792 (1997)). Thus, antigens on viral particles that are organized in an ordered and repetitive array are highly immunogenic since they can directly activate B cells.

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In addition to strong B cell responses, viral particles are also able to induce the generation of a cytotoxic T cell response, another crucial arm of the immune system. These cytotoxic T cells are particularly important for the elimination of non-cytopathic viruses such as HIV or hepatitis B virus and for the eradication of tumors. Cytotoxic T cells do not recognize native antigens but rather recognize their degradation products in association with MHC class I molecules (Townsend & Bodmer, *Ann. Rev. Immunol.* 7:601-624 (1989)). Macrophages and dendritic cells are able to take up and process exogenous viral particles (but not their soluble, isolated components) and present the generated degradation product to cytotoxic T cells, leading to their activation and proliferation (Kovacsovics-Bankowski *et al.*, *Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993); Bachmann *et al.*, *Eur. J. Immunol.* 26:2595-2600 (1996)).

Viral particles as antigens exhibit two advantages over their isolated components: (1) Due to their highly repetitive surface structure, they are able to directly activate B cells, leading to high antibody titers and long-lasting B cell memory; and (2) Viral particles but not soluble proteins are able to induce a cytotoxic T cell response, even if the viruses are non-infectious and adjuvants are absent.

Several new vaccine strategies exploit the inherent immunogenicity of viruses. Some of these approaches focus on the particulate nature of the virus particle; for example *see* Harding, C.V. and Song, R., (*J. Immunology* 153:4925 (1994)), which discloses a vaccine consisting of latex beads and antigen; Kovacsovics-Bankowski, M., *et al.* (*Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993)), which discloses a vaccine consisting of iron oxide beads and antigen; U.S. Patent No: 5,334,394 to Kossovsky, N., *et al.*, which discloses core particles coated with antigen; U.S. Patent No. 5,871,747, which discloses synthetic polymer particles carrying on the surface one or more proteins covalently bonded thereto; and a core particle with a non-covalently bound coating, which at least partially covers the surface of said core particle, and at least one biologically active agent in contact with said coated core particle (*see, e.g.*, WO/94/15585).

However, a disadvantage of these viral mimicry systems is that they are not able to recreate the ordered presentation of antigen found on the viral surface. Antigens coupled to a surface in a random orientation are found to induce CTL response and no or only weak B-cell response. For an efficient vaccine, both arms of the immune system have to be strongly activated, as described above and in Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235 (1997).

In another example, recombinant viruses are being utilized for antigen delivery. Filamentous phage virus containing an antigen fused to a capsid protein has been found to be highly immunogenic (*see* Perham R.N., *et al.*, *FEMS Microbiol. Rev.* 17:25-31 (1995); Willis *et al.*, *Gene* 128:85-88 (1993); Minenkova *et al.*, *Gene* 128:85-88 (1993)). However, this system is limited to very small peptides (5 or 6 amino acid residues) when the fusion protein is expressed at a high level (Iannolo *et al.*, *J. Mol. Biol.* 248:835-844 (1995)) or limited to the low level expression of larger proteins (de la Cruz *et al.*, *J. Biol. Chem.* 263:4318-4322 (1988)). For small peptides, so far only the CTL response is observed and no or only weak B-cell response.

In yet another system, recombinant alphaviruses are proposed as a means of antigen delivery (*see* U.S. Patent Nos. 5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). Problems with the recombinant virus systems described so far include a low density expression of the heterologous protein on the viral surface and/or the difficulty of successfully and repeatedly creating a new and different recombinant viruses for different applications.

In a further development, virus-like particles (VLPs) are being exploited in the area of vaccine production because of both their structural properties and their non-infectious nature. VLPs are supermolecular structures built in a symmetric manner from many protein molecules of one or more types. They lack the viral genome and, therefore, are noninfectious. VLPs can often be produced in large quantities by heterologous expression and can be easily be purified.

Examples of VLPs include the capsid proteins of hepatitis B virus (Ulrich, *et al.*, *Virus Res.* 50:141-182 (1998)), measles virus (Warnes, *et al.*, *Gene*



160:173-178 (1995)), Sindbis virus, rotavirus (US Patent Nos. 5,071,651 and 5,374,426), foot-and-mouth-disease virus (Twomey, *et al.*, Vaccine 13: 1603-1610, (1995)), Norwalk virus (Jiang, X., *et al.*, Science 250:1580-1583 (1990); Matsui, S.M., *et al.*, J. Clin. Invest. 87:1456-1461 (1991)), the retroviral GAG protein (PCT Patent Appl. No. WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291) and human papilloma virus (WO 98/15631). In some instances, recombinant DNA technology may be utilized to fuse a heterologous protein to a VLP protein (Kratz, P.A., *et al.*, Proc. Natl. Acad. Sci. USA 96: 19151920 (1999)).

Thus, there is a need in the art for the development of new and improved vaccines that promote a strong CTL and B-cell immune response as efficiently as natural pathogens.

### *Summary of the Invention*

The invention provides a versatile new technology that allows production of particles coated with any desired antigen. The technology allows the creation of highly efficient vaccines against infectious diseases and for the creation of vaccines for the treatment of allergies and cancers.

In a first embodiment, the invention provides a novel composition comprising (A) a non-natural molecular scaffold and (B) an antigen or antigenic determinant.

The non-natural molecular scaffold comprises (i) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a core particle of natural origin; and (ii) an organizer comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

The antigen or antigenic determinant has at least one second attachment site which is selected from the group consisting of (i) an attachment site not

naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant.

The invention provides for an ordered and repetitive antigen array through an association of the second attachment site to the first attachment site by way of at least one non-peptide bond. Thus, the antigen or antigenic determinant and the non-natural molecular scaffold are brought together through this association of the first and the second attachment site to form an ordered and repetitive antigen array.

In another embodiment, the core particle of the aforementioned composition comprises a virus, a virus-like particle, a bacteriophage, a viral capsid particle or a recombinant form thereof. Alternatively, the core particle may be a synthetic polymer or a metal.

In a particular embodiment, the organizer may comprise at least one first attachment site. The first and the second attachment sites are particularly important elements of the composition of the invention. In various embodiments of the invention, the first and/or the second attachment site may be an antigen and an antibody or antibody fragment thereto; biotin and avidin; streptavidin and biotin; a receptor and its ligand; a ligand-binding protein and its ligand; interacting leucine zipper polypeptides; an amino group and a chemical group reactive thereto; a carboxyl group and a chemical group reactive thereto; a sulfhydryl group and a chemical group reactive thereto; or a combination thereof.

In a more preferred embodiment, the invention provides the coupling of almost any antigen of choice to the surface of a virus, bacteriophage, virus-like particle or viral capsid particle. By bringing an antigen into a quasi-crystalline 'virus-like' structure, the invention exploits the strong antiviral immune reaction of a host for the production of a highly efficient immune response, *i.e.*, a vaccination, against the displayed antigen.

In one preferred embodiment, the core particle may be selected from the group consisting of: recombinant proteins of Rotavirus, recombinant proteins of Norwalkvirus, recombinant proteins of Alphavirus, recombinant proteins of Foot

and Mouth Disease virus, recombinant proteins of Retrovirus, recombinant proteins of Hepatitis B virus, recombinant proteins of Tobacco mosaic virus, recombinant proteins of Flock House Virus, and recombinant proteins of human Papillomavirus.

5           In another preferred embodiment, the antigen may be selected from the group consisting of: (1) a protein suited to induce an immune response against cancer cells; (2) a protein suited to induce an immune response against infectious diseases; (3) a protein suited to induce an immune response against allergens; and (4) a protein suited to induce an immune response in farm animals.

10           In a particularly preferred embodiment of the invention, the first attachment site and/or the second attachment site comprise an interacting leucine zipper polypeptide. In most preferred embodiment, the first attachment site and/or the second attachment site are selected from the group comprising: (1) the *JUN* leucine zipper protein domain; and (2) the *FOS* leucine zipper protein domain.

15           In another preferred embodiment, the first attachment site and/or the second attachment site are selected from the group comprising: (1) a genetically engineered lysine residue and (2) a genetically engineered cysteine residue, two residues that may be chemically linked together.

20           Other embodiments of the invention include processes for the production of the compositions of the invention and a methods of medical treatment using said compositions.

25           It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

### *Brief Description of the Drawings*

**Figure 1** Western blot demonstrating the production of viral particles containing the E2-JUN fusion protein using the pCYTts::E2JUN expression vector.

5        **Figure 2** Western blot demonstrating the production of viral particles containing the E2-JUN fusion protein expressed from pTES'2J::E2JUN expression vector.

**Figure 3** Western dot blot demonstrating bacterial and eukaryotic expression of the FOS-hgh antigen.

10        **Figure 4** Expression of HBcAg-JUN in *E. coli* cells.

**Figure 5** Western blot demonstrating that HBcAg-JUN is soluble in *E. coli* lysates.

**Figure 6** SDS-PAGE analysis of enrichment of HBcAg-JUN capsid particles on a sucrose density gradient.

15        **Figure 7** Non-reducing SDS-PAGE analysis of the coupling of hGH-FOS and HBcAg-JUN particles.

### *Detailed Description of the Preferred Embodiments*

#### *1. Definitions*

20        The following definitions are provided to clarify the subject matter which the inventors consider to be the present invention.

**Alphavirus:** As used herein, the term "alphavirus" refers to any of the RNA viruses included within the genus *Alphavirus*. Descriptions of the members of this genus are contained in Strauss and Strauss, *Microbiol. Rev.*, 58:491-562 (1994). Examples of alphaviruses include Aura virus, Bebaru virus, Cabassou virus, Chikungunya virus, Easter equine encephalomyelitis virus, Fort morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus,

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Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Trinita virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Sindbis virus (SIN), Semliki forest virus (SFV), Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

5           **Antigen:** As used herein, the term "antigen" is a molecule capable of being bound by an antibody. An antigen is additionally capable of inducing a humoral immune response and/or cellular immune response leading to the production of B- and/or T-lymphocytes. An antigen may have one or more epitopes (B- and T- epitopes). The specific reaction referred to above is meant  
10 to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

**Antigenic determinant:** As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by  
15 either B- or T-lymphocytes. B-lymphocytes respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediator of cellular immunity. Thus, antigenic determinants or epitopes are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptors.

20           **Association:** As used herein, the term "association" as it applies to the first and second attachment sites, is used to refer to at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof.

**Attachment Site, First:** As used herein, the phrase "first attachment site" refers to an element of the "organizer", itself bound to the core particle in a non-  
25 random fashion, to which the second attachment site located on the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin,  
30 metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a

chemically reactive group thereof. Multiple first attachment sites are present on the surface of the non-natural molecular scaffold in a repetitive configuration.

**Attachment Site, Second:** As used herein, the phrase "second attachment site" refers to an element associated with the antigen or antigenic determinant to which the first attachment site of the "organizer" located on the surface of the non-natural molecular scaffold may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant.

**Core particle:** As used herein, the term "core particle" refers to a rigid structure with an inherent repetitive organization that provides a foundation for attachment of an "organizer". A core particle as used herein may be the product of a synthetic process or the product of a biological process.

**Cis-acting:** As used herein, the phrase "cis-acting" sequence refers to nucleic acid sequences to which a replicase binds to catalyze the RNA-dependent replication of RNA molecules. These replication events result in the replication of the full-length and partial RNA molecules and, thus, the alphavirus subgenomic promoter is also a "cis-acting" sequence. *Cis-acting* sequences may be located at or near the 5' end, 3' end, or both ends of a nucleic acid molecule, as well as internally.

**Fusion:** As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, *i.e.*, insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.

**Heterologous sequence:** As used herein, the term "heterologous sequence" refers to a second nucleotide sequence present in a vector of the

invention. The term "heterologous sequence" also refers to any amino acid or RNA sequence encoded by a heterologous DNA sequence contained in a vector of the invention. Heterologous nucleotide sequences can encode proteins or RNA molecules normally expressed in the cell type in which they are present or molecules not normally expressed therein (*e.g.*, Sindbis structural proteins).

**Isolated:** As used herein, when the term "isolated" is used in reference to a molecule, the term means that the molecule has been removed from its native environment. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated." Further, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Isolated RNA molecules include *in vivo* or *in vitro* RNA replication products of DNA and RNA molecules. Isolated nucleic acid molecules further include synthetically produced molecules. Additionally, vector molecules contained in recombinant host cells are also isolated. Thus, not all "isolated" molecules need be "purified."

**Immunotherapeutic:** As used herein, the term "immunotherapeutic" is a composition for the treatment of diseases or disorders.. More specifically, the term is used to refer to a method of treatment for allergies or a method of treatment for cancer.

**Individual:** As used herein, the term "individual" refers to multicellular organisms and includes both plants and animals. Preferred multicellular organisms are animals, more preferred are vertebrates, even more preferred are mammals, and most preferred are humans.

**Low or undetectable:** As used herein, the phrase "low or undetectable," when used in reference to gene expression level, refers to a level of expression which is either significantly lower than that seen when the gene is maximally induced (*e.g.*, at least five fold lower) or is not readily detectable by the methods used in the following examples section.

**Lectin:** As used herein, proteins obtained particularly from the seeds of leguminous plants, but also from many other plant and animal sources, that have binding sites for specific mono- or oligosaccharides. Examples include concanavalin A and wheat-germ agglutinin, which are widely used as analytical and preparative agents in the study of glycoproteins.

**Natural origin:** As used herein, the term "natural origin" means that the whole or parts thereof are not synthetic and exist or are produced in nature.

**Non-natural:** As used herein, the term generally means not from nature, more specifically, the term means from the hand of man.

**Non-natural origin:** As used herein, the term "non-natural origin" generally means synthetic or not from nature; more specifically, the term means from the hand of man.

**Non-natural molecular scaffold:** As used herein, the phrase "non-natural molecular scaffold" refers to any product made by the hand of man that may serve to provide a rigid and repetitive array of first attachment sites. Ideally but not necessarily, these first attachment sites are in a geometric order. The non-natural molecular scaffold may be organic or non-organic and may be synthesized chemically or through a biological process, in part or in whole. The non-natural molecular scaffold is comprised of: (a) a core particle, either of natural or non-natural origin; and (b) an organizer, which itself comprises at least one first attachment site and is connected to a core particle by at least one covalent bond. In a particular embodiment, the non-natural molecular scaffold may be a virus, virus-like particle, a virus capsid particle, a phage, a recombinant form thereof, or synthetic particle.

**Ordered and repetitive antigen or antigenic determinant array:** As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a uniform spacial arrangement of the antigens or antigenic determinants with respect to the scaffold. In one embodiment of the invention, the repeating pattern may be a geometric pattern. An ideal ordered and repetitive



antigen or antigenic determinant array will possess a strictly repetitive paracrystalline order of antigen or antigenic determinant with a spacing of 5 to 15 nanometers.

**Organizer:** As used herein, the term "organizer" is used to refer to an element bound to a core particle in a non-random fashion that provides a nucleation site for creating an ordered and repetitive antigen array. An organizer is any element comprising at least one first attachment site that is bound to a core particle by at least one covalent bond. An organizer may be a protein, a polypeptide, a peptide, an amino acid (*i.e.*, a residue of a protein, a polypeptide or peptide), a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof.

**Permissive temperature:** As used herein, the phrase "permissive temperature" refers to temperatures at which an enzyme has relatively high levels of catalytic activity.

**Purified:** As used herein, when the term "purified" is used in reference to a molecule, it means that the concentration of the molecule being purified has been increased relative to molecules associated with it in its natural environment. Naturally associated molecules include proteins, nucleic acids, lipids and sugars but generally do not include water, buffers, and reagents added to maintain the integrity or facilitate the purification of the molecule being purified. For example, even if mRNA is diluted with an aqueous solvent during oligo dT column chromatography, mRNA molecules are purified by this chromatography if naturally associated nucleic acids and other biological molecules do not bind to the column and are separated from the subject mRNA molecules.

**Receptor:** As used herein, the term "receptor" refers to proteins or glycoproteins or fragments thereof capable of interacting with another molecule, called the ligand. The ligand may belong to any class of biochemical or chemical compounds. The receptor need not necessarily be a membrane-bound protein.

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Soluble protein, like *e.g.* maltose binding protein or retinol binding protein are receptors as well.

**Residue.** As used herein, the term "residue" is meant to mean a specific amino acid in a polypeptide backbone or side chain.

5       **Temperature-sensitive:** As used herein, the phrase "temperature-sensitive" refers to an enzyme which readily catalyzes a reaction at one temperature but catalyzes the same reaction slowly or not at all at another temperature. An example of a temperature-sensitive enzyme is the replicase protein encoded by the pCYTts vector, which has readily detectable replicase  
10 activity at temperatures below 34°C and has low or undetectable activity at 37°C.

**Transcription:** As used herein, the term "transcription" refers to the production of RNA molecules from DNA templates catalyzed by RNA polymerases.

15       **Recombinant host cell:** As used herein, the term "recombinant host cell" refers to a host cell into which one or more nucleic acid molecules of the invention have been introduced.

20       **Recombinant virus:** As used herein, the phrase "recombinant virus" refers to a virus that is genetically modified by the hand of man. The phrase covers any virus known in the art. More specifically, the phrase refers to an alphavirus genetically modified by the hand of man, and most specifically, the phrase refers to a Sinbis virus genetically modified by the hand of man.

25       **Restrictive temperature:** As used herein, the phrase "restrictive temperature" refers to temperatures at which an enzyme has low or undetectable levels of catalytic activity. Both "hot" and "cold" sensitive mutants are known and, thus, a restrictive temperature may be higher or lower than a permissive temperature.

**RNA-dependent RNA replication event:** As used herein, the phrase "RNA-dependent RNA replication event" refers to processes which result in the formation of an RNA molecule using an RNA molecule as a template.

**RNA-Dependent RNA polymerase:** As used herein, the phrase "RNA-Dependent RNA polymerase" refers to a polymerase which catalyzes the production of an RNA molecule from another RNA molecule. This term is used herein synonymously with the term "replicase."

5       **Untranslated RNA:** As used herein, the phrase "untranslated RNA" refers to an RNA sequence or molecule which does not encode an open reading frame or encodes an open reading frame, or portion thereof, but in a format in which an amino acid sequence will not be produced (*e.g.*, no initiation codon is present). Examples of such molecules are tRNA molecules, rRNA molecules, and  
10       ribozymes.

**Vector:** As used herein, the term "vector" refers to an agent (*e.g.*, a plasmid or virus) used to transmit genetic material to a host cell. A vector may be composed of either DNA or RNA.

15       **one, a, or an:** When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

## 2.       *Compositions of Ordered and Repetitive Antigen or Antigenic Determinant Arrays and Methods to Make the Same*

The disclosed invention provides compositions comprising an ordered and repetitive antigen or antigenic determinant. Furthermore, the invention  
20       conveniently enables the practitioner to construct ordered and repetitive antigen or antigenic determinant arrays for various treatment purposes, which includes the prevention of infectious diseases, the treatment of allergies and the treatment of cancers.

25       Compositions of the invention essentially comprise two elements: (1) a non-natural molecular scaffold; and (2) an antigen or antigenic determinant with at least one second attachment site capable of association through at least one non-peptide bond to said first attachment site.

The non-natural molecular scaffold comprises (a) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a

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core particle of natural origin; and (b) an organizer comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

5 The antigen or antigenic determinant has at least one second attachment site which is selected from the group consisting of (a) an attachment site not naturally occurring with said antigen or antigenic determinant; and (b) an attachment site naturally occurring with said antigen or antigenic determinant.

10 The invention provides for an ordered and repetitive antigen array through an association of the second attachment site to the first attachment site by way of at least one non-peptide bond. Thus, the antigen or antigenic determinant and the non-natural molecular scaffold are brought together through this association of the first and the second attachment site to form an ordered and repetitive antigen array.

15 The practitioner may specifically design the antigen or antigenic determinant and the second attachment site such that the arrangement of all the antigens or antigenic determinants bound to the non-natural molecular scaffold will be uniform. For example, one may place a single second attachment site on the antigen or antigenic determinant at the carboxyl or amino terminus, thereby ensuring through design that all antigen or antigenic determinant molecules that are attached to the non-natural molecular scaffold are positioned in a uniform way. Thus, the invention provides a convenient means of placing any antigen or antigenic determinant onto a non-natural molecular scaffold in a defined order and repetition.

25 As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning, polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies are well known to those skilled in the art and may be conveniently found in published laboratory methods manuals (*e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring

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Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)). Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth. Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., "Protein Purification Principles and Practice," 3<sup>rd</sup> ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

*A. Construction of a Non-natural Molecular Scaffold*

One element in the composition of the invention is a non-natural, molecular scaffold comprising a core particle and an organizer. As used herein, the phrase "non-natural molecular scaffold" refers to any product made by the hand of man that may serve to provide a rigid and repetitive array of first attachment sites. More specifically, the non-natural molecular scaffold comprises (a) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a core particle of natural origin; and (b) an organizer comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

As will be readily apparent to those skilled in the art, the core particle of the non-natural molecular scaffold of the invention is not limited to any specific form. The core particle may be organic or non-organic and may be synthesized chemically or through a biological process.

In one embodiment, a non-natural core particle may be a synthetic polymer, a lipid micelle or a metal. Such core particles are known in the art, providing a basis from which to build the novel non-natural molecular scaffold of the invention. By way of example, synthetic polymer or metal core particles

are described in U.S. Patent No. 5,770,380, which discloses the use of a calixarene organic scaffold to which is attached a plurality of peptide loops in the creation of an 'antibody mimic', and U.S. Patent No. 5,334,394 describes nanocrystalline particles used as a viral decoy that are composed of a wide variety of inorganic materials, including metals or ceramics. Preferred metals in this embodiment include chromium, rubidium, iron, zinc, selenium, nickel, gold, silver, platinum. Preferred ceramic materials in this embodiment include silicon dioxide, titanium dioxide, aluminum oxide, ruthenium oxide and tin oxide. The core particles of this embodiment may be made from organic materials including carbon (diamond). Preferred polymers include polystyrene, nylon and nitrocellulose. For this type of nanocrystalline particle, particles made from tin oxide, titanium dioxide or carbon (diamond) are particularly preferred. A lipid micelle may be prepared by any means known in the art. For example micelles may be prepared according to the procedure of Baiselle and Millar (Baiselle, C.J. and Millar, D.B., *Biophys. Chem.* 4:355-361 (1975)) or Corti *et al.* (Corti, M., Degriorgio, V., Sonnino, S., Ghidoni R., Masserini, M. and Tettamanti, G., *Chem. Phys. Lipids* 38: 197-214 (1981)) or Lopez *et al.* (Lopez, O. de la Maza, A., Coderch, L., Lopez-Iglesias, C., Wehrli, E. and Parra, J.L., *FEBS Lett.* 426: 314-318 (1998)) or Topchieva and Karezin (Topchieva, I. and Karaezin, K., *J. Colloid Interface Sci.* 213: 29-35 (1999)) or Morein *et al.*, (Morein, B., Sundquist, B., Hoglund, S., Dalsgaard K. and Osterhaus, A., *Nature* 308: 457-60 (1984)), which are all incorporated herein by reference.

The core particle may also be produced through a biological process, which may be natural or non-natural. By way of example, this type of embodiment may includes a core particle comprising a virus, virus-like particle, a phage, a viral capsid particle or a recombinant form thereof. In a more specific embodiment, the core particle may comprise recombinant proteins of Rotavirus, recombinant proteins of Norwalkvirus, recombinant proteins of Alphavirus, recombinant proteins of Foot and Mouth Disease virus, recombinant proteins of Retrovirus, recombinant proteins of Hepatitis B virus, recombinant proteins of

Tobacco mosaic virus, recombinant proteins of Flock House Virus, and recombinant proteins of human Papillomavirus.

Whether natural or non-natural, the core particle of the invention is characterized by comprising organizer that is attached to the natural or non-natural core particle by at least one covalent bond. The organizer is an element  
5 bound to a core particle in a non-random fashion that provides a nucleation site for creating an ordered and repetitive antigen array. Ideally, but not necessarily, the organizer is associated with the core particle in a geometric order. Minimally, the organizer comprises a first attachment site.

10 As previously stated, the organizer may be any element comprising at least one first attachment site that is bound to a core particle by at least one covalent bond. An organizer may be a protein, a polypeptide, a peptide, an amino acid (*i.e.*, a residue of a protein, a polypeptide or peptide), a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or  
15 compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. In a more specific embodiment, the organizer may comprise a first attachment site comprising an antigen, an antibody or antibody fragment, biotin, avidin, streptavidin, a receptor, a receptor ligand, a ligand, a ligand-binding  
20 protein, an interacting leucine zipper polypeptide, an amino group, a chemical group reactive to an amino group; a carboxyl group, chemical group reactive to a carboxyl group, a sulfhydryl group, a chemical group reactive to a sulfhydryl group, or a combination thereof.

In a preferred embodiment, the core particle of the non-natural molecular  
25 scaffold comprises a virus, a bacteriophage, a virus-like particle, a viral capsid particle or a recombinant form thereof. Any virus known in the art having an ordered and repetitive coat and/or core protein structure may be selected as a non-natural molecular scaffold of the invention; examples of suitable viruses include: sindbis and other alphaviruses; vesicular stomatitis virus; rhabdo-, (*e.g.* vesicular  
30 stomatitis virus), picorna-, toga-, orthomyxo-, polyoma-, parvovirus, rotavirus,

norwalkvirus, foot and mouth disease virus, a retrovirus, hepatitis B virus, tobacco mosaic virus, flock house virus, human papillomavirus (for example, see Table 1 in Bachman, M.F. and Zinkernagel, R.M., *Immunol. Today* 17:553-558 (1996)).

5 In one embodiment, the invention utilizes genetic engineering of a virus to create a fusion between an ordered and repetitive viral envelope protein and an organizer comprising a heterologous protein, peptide, antigenic determinant or a reactive amino acid residue of choice. Other genetic manipulations known to those in the art may be included in the construction of the non-natural molecular  
10 scaffold; for example, it may be desirable to restrict the replication ability of the recombinant virus through genetic mutation. The viral protein selected for fusion to the organizer (i.e., first attachment site) protein should have an organized and repetitive structure, more preferably a paracrystalline organization optimally with a spacing of 5-15nm on the surface of the virus. The creation of this type of  
15 fusion protein will result in multiple, ordered and repetitive organizers on the surface of the virus. Thus, the ordered and repetitive organization of the first attachment sites resulting therefrom will reflect the normal organization of the native viral protein.

As will be discussed in more detail herein, in a preferred embodiment of  
20 the invention, the scaffold is a recombinant alphavirus, and more specifically, a recombinant Sinbis virus. Alphaviruses are positive stranded RNA viruses that replicate their genomic RNA entirely in the cytoplasm of the infected cell and without a DNA intermediate (Strauss, J. and Strauss, E., *Microbiol. Rev.* 58:491-562 (1994)). Several members of the alphavirus family, Sindbis (Xiong, C. *et al.*, *Science* 243:1188-1191 (1989); Schlesinger, S., *Trends Biotechnol.* 11:18-22 (1993)), Semliki Forest Virus (SFV) (Liljeström, P. & Garoff, H., *Bio/Technology* 9:1356-1361 (1991)) and others (Davis, N.L. *et al.*, *Virology* 171:189-204 (1989)), have received considerable attention for use as virus-based expression  
25 vectors for a variety of different proteins (Lundstrom, K., *Curr. Opin. Biotechnol.* 8:578-582 (1997); Liljeström, P., *Curr. Opin. Biotechnol.* 5:495-500 (1994)) and  
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as candidates for vaccine development. Recently, a number of patents have issued directed to the use of alphaviruses for the expression of heterologous proteins and the development of vaccines (*see* U.S. Patent Nos. 5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). The construction of the  
5 alphaviral scaffold of the invention may be done by means generally known in the art of recombinant DNA technology, as described by the aforementioned articles, which are incorporated herein by reference.

A variety of different recombinant host cells can be utilized to produce a viral-based core particle for antigen or antigenic determinant attachment. For  
10 example, Alphaviruses are known to have a wide host range; Sindbis virus infects cultured mammalian, reptilian, and amphibian cells, as well as some insect cells (Clark, H., *J. Natl. Cancer Inst.* 51:645 (1973); Leake, C., *J. Gen. Virol.* 35:335 (1977); Stollar, V. in *THE TOGAVIRUSES*, R. W. Schlesinger, Ed., Academic Press, (1980), pp.583-621). Thus, numerous recombinant host cells can be used in the  
15 practice of the invention. BHK, COS, Vero, HeLa and CHO cells are particularly suitable for the production of heterologous proteins because they have the potential to glycosylate heterologous proteins in a manner similar to human cells (Watson, E. *et al.*, *Glycobiology* 4:227, (1994)) and can be selected (Zang, M. *et al.*, *Bio/Technology* 13:389 (1995)) or genetically engineered (Renner W. *et al.*, *Biotech. Bioeng.* 4:476 (1995); Lee K. *et al.* *Biotech. Bioeng.* 50:336 (1996)) to  
20 grow in serum-free medium, as well as in suspension.

Introduction of the polynucleotide vectors into host cells can be effected by methods described in standard laboratory manuals (*see, e.g.*, Sambrook, J. *et al.*, eds., *MOLECULAR CLONING, A LABORATORY MANUAL*. 2nd. edition, Cold  
25 Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Chapter 9; Ausubel, F. *et al.*, eds., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John H. Wiley & Sons, Inc. (1997), Chapter 16), including methods such as electroporation, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, transduction, scrape loading,  
30 ballistic introduction, and infection. Methods for the introduction of exogenous

DNA sequences into host cells are discussed in Felgner, P. *et al.*, U.S. Patent No. 5,580,859.

Packaged RNA sequences can also be used to infect host cells. These packaged RNA sequences can be introduced to host cells by adding them to the culture medium. For example, the preparation of non-infective alphaviral particles is described in a number of sources, including "Sindbis Expression System", Version C, (*Invitrogen* Catalog No. K750-1).

When mammalian cells are used as recombinant host cells for the production of viral-based core particles, these cells will generally be grown in tissue culture. Methods for growing cells in culture are well known in the art (*see, e.g.*, Celis, J., ed., *CELL BIOLOGY*, Academic Press, 2<sup>nd</sup> edition, (1998); Sambrook, J. *et al.*, eds., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John H. Wiley & Sons, Inc. (1997); Freshney, R., *CULTURE OF ANIMAL CELLS*, Alan R. Liss, Inc. (1983)).

As will be understood by those in the art, the first attachment site may be or be a part of any suitable protein, polypeptide, sugar, polynucleotide, peptide (amino acid), natural or synthetic polymer, a secondary metabolite or combination thereof that may serve to specifically attach the antigen or antigenic determinant of choice to the scaffold. In one embodiment, the attachment site is a protein or peptide that may be selected from those known in the art. For example, the first attachment site may selected from the following group: a ligand, a receptor, a lectin, avidin, streptavidin, biotin, an epitope such as an HA or T7 tag, Myc, Max, immunoglobulin domains and any other amino acid sequence known in the art that would be useful as a first attachment site.

It should be further understood by those in the art that with another embodiment of the invention, the first attachment site may be created secondarily to the organizer (i.e., protein or polypeptide) utilized in constructing the in-frame fusion to the capsid protein. For example, a protein may be utilized for fusion to

the envelope protein with an amino acid sequence known to be glycosylated in a specific fashion, and the sugar moiety added as a result may then serve at the first attachment site of the viral scaffold by way of binding to a lectin serving as the secondary attachment site of an antigen. Alternatively, the organizer sequence  
5 may be biotinylated *in vivo* and the biotin moiety may serve as the first attachment site of the invention, or the organizer sequence may be subjected to chemical modification of distinct amino acid residues *in vitro*, the modification serving as the first attachment site.

One specific embodiment of the invention utilizes the Sinbis virus. The  
10 Sinbis virus RNA genome is packaged into a capsid protein that is surrounded by a lipid bilayer containing three proteins called E1, E2, and E3. These so-called envelope proteins are glycoproteins, and the glycosylated portions are located on the outside of the lipid bilayer, where complexes of these proteins form the "spikes" that can be seen in electron micrographs to project outward from the  
15 surface of the virus. In a preferred embodiment of the invention, the first attachment site is selected to be the *JUN* or *FOS* leucine zipper protein domain that is fused in frame to the E2 envelope protein. However, it will be clear to all individuals in the art that other envelope proteins may be utilized in the fusion protein construct for locating the first attachment site in the scaffold of the  
20 invention.

In a most preferred embodiment of the invention, the first attachment site is selected to be the *JUN-FOS* leucine zipper protein domain that is fused in frame to the Hepatitis B capsid (core) protein. However, it will be clear to all  
25 individuals in the art that other viral capsid proteins may be utilized in the fusion protein construct for locating the first attachment site in the scaffold of the invention.

In another preferred embodiment of the invention, the first attachment site is selected to be a lysine or cysteine residue that is fused in frame to the Hepatitis core (capsid) protein. However, it will be clear to all individuals in the art that

other viral capsid or virus-like particles may be utilized in the fusion protein construct for locating the first attachment in the scaffold of the invention.

Example 1 is provided to demonstrate the construction of an in-frame fusion protein between the Sinbis virus E2 envelope protein and the *JUN* leucine zipper protein domain using the pTE5'2J vector of Hahn *et al.* (*Proc. Natl. Acad. Sci. USA* 89:2679-2683 (1992)). The *JUN* amino acid sequence utilized for the first attachment site is the following:

CGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNHVGC  
(SEQ ID NO:59)

In this instance, the anticipated second attachment site on the antigen would be the *FOS* leucine zipper protein domain and the amino acid sequence would be the following:

CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC (SEQ  
ID NO:60)

These sequences are derived from the transcription factors *JUN* and *FOS*, each flanked with a short sequence containing a cysteine residue on both sides. These sequences are known to interact with each other. The original hypothetical structure proposed for the *JUN-FOS* dimer assumed that the hydrophobic side chains of one monomer interdigitate with the respective side chains of the other monomer in a zipper-like manner (Landschulz *et al.*, *Science* 240:1759-1764 (1988)). However, this hypothesis proved to be wrong, and these proteins are known to form an  $\alpha$ -helical coiled coil (O'Shea *et al.*, *Science* 243:538-542 (1989); O'Shea *et al.*, *Cell* 68:699-708 (1992); Cohen & Parry, *Trends Biochem. Sci.* 11:245-248 (1986)). Thus, the term "leucine zipper" is frequently used to refer to these protein domains for more historical than structural reasons. Throughout this patent, the term "leucine zipper" is used to refer to the sequences depicted above or sequences essentially similar to the ones depicted above. The terms *JUN* and *FOS* are used for the respective leucine zipper domains rather than the entire *JUN* and *FOS* proteins.

In one embodiment, the invention provides for the production of a Sinbis virus E2-*JUN* scaffold using the pCYTts expression system (US Patent Application Appl. No. 60/079,562; Filed March 27,1998). The pCYTts expression system provides novel expression vectors which permit tight regulation of gene expression in eucaryotic cells. The DNA vectors of this system are transcribed to form RNA molecules which are then replicated by a temperature-sensitive replicase to form additional RNA molecules. The RNA molecules produced by replication contain a nucleotide sequence which may be translated to produce a protein of interest or which encode one or more untranslated RNA molecules. Thus the expression system enables the production of recombinant Sinbis virus particles.

Example 2 provides details on the production of the E2-*JUN* Sinbis non-natural, molecular scaffold of the invention. Additionally provided in Example 3 is another method for the production of recombinant E2-*JUN* Sinbis virus scaffold using the pTE5'2JE2:*JUN* vector produced in Example 1. Thus the invention provides two means, the pCYTts expression system (Example 2) and the pTE5'2J vector system (Example 3) by which recombinant Sinbis virus E2-*JUN* non-natural, molecular scaffold may be produced. An analysis of viral particles produced in each system is proved in Figure 1 and Figure 2.

As previously stated, the invention includes viral-based core particles which comprise a virus, virus-like particle, a phage, a viral capsid particle or a recombinant form thereof. Skilled artisans have the knowledge to produce such core particles and attach organizers thereto. By way of providing other examples, the invention provides herein for the production of hepatitis B virus-like particles and measles viral capsid particles as core particles (Examples 17 to 22). In such an embodiment, the *JUN* leucine zipper protein domain or *FOS* leucine zipper protein domain may be used as an organizer, and hence as a first attachment site, for the non-natural molecular scaffold of the invention.

Examples 23-29 provide details of the production of Hepatitis B core particles carrying an in-frame fused peptide with a reactive lysine residue and

antigens carrying a genetically fused cysteine residue, as first and second attachment site, respectively.

***B. Construction of an Antigen or Antigenic Determinant with a Second Attachment Site***

5           The second element in the composition of the invention is an antigen or antigenic determinant possessing at least one second attachment site capable of association through at least one non-peptide bond to the first attachment site of the non-natural molecular scaffold. The invention provides for compositions that vary according to the antigen or antigenic determinant selected in consideration  
10 of the desired therapeutic effect. Other compositions are provided by varying the molecule selected for the second attachment site.

          Antigens of the invention may be selected from the group consisting of the following: (a) proteins suited to induce an immune response against cancer cells; (b) proteins suited to induce an immune response against infectious diseases; (c)  
15 proteins suited to induce an immune response against allergens, and (d) proteins suited to induce an immune response in farm animals.

          In one specific embodiment of the invention, the antigen or antigenic determinant is one that is useful for the prevention of infectious disease. Such treatment will be useful to treat a wide variety of infectious diseases affecting a  
20 wide range of hosts, *e.g.*, human, cow, sheep, pig, dog, cat, other mammalian species and non-mammalian species as well. Treatable infectious diseases are well known to those skilled in the art, examples include infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, etc.; or infections of bacterial etiology such  
25 as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc. Thus, antigens or antigenic determinants selected for the compositions of the invention will be well known to those in the medical art; examples of antigens or antigenic determinants include the following: the HIV antigens gp140 and gp160;

the influenza antigens hemagglutinin and neuraminidase, hepatitis B surface antigen, circumsporozoite protein of malaria.

In another specific embodiment, the compositions of the invention are an immunotherapeutic that may be used for the treatment of allergies or cancer.

5           The selection of antigens or antigenic determinants for the composition and method of treatment for allergies would be known to those skilled in the medical art treating such disorders; representative examples of this type of antigen or antigenic determinant include the following: bee venom phospholipase A<sub>2</sub>, Bet v I (birch pollen allergen), 5 Dol m V (white-faced hornet venom allergen), Der p I (House dust mite allergen).

10           The selection of antigens or antigenic determinants for the composition and method of treatment for cancer would be known to those skilled in the medical art treating such disorders; representative examples of this type of antigen or antigenic determinant include the following: Her2 (breast cancer), GD2 (neuroblastoma), EGF-R (malignant glioblastoma), CEA (medullary thyroid cancer), CD52 (leukemia).

15           In a particular embodiment of the invention, the antigen or antigenic determinant is selected from the group consisting of: (a) a recombinant protein of HIV, (b) a recombinant protein of Influenza virus, (c) a recombinant protein of Hepatitis C virus, (d) a recombinant protein of Toxoplasma, (e) a recombinant protein of Plasmodium falciparum, (f) a recombinant protein of Plasmodium vivax, (g) a recombinant protein of Plasmodium ovale, (h) a recombinant protein of Plasmodium malariae, (i) a recombinant protein of breast cancer cells, (j) a recombinant protein of kidney cancer cells, (k) a recombinant protein of prostate cancer cells, (l) a recombinant protein of skin cancer cells, (m) a recombinant protein of brain cancer cells, (n) a recombinant protein of leukemia cells, (o) a recombinant protein of prostate cancer cells, (p) a recombinant protein of bee sting allergy, (q) a recombinant protein of nut allergy, (r) a recombinant protein of food allergies, recombinant proteins of asthma, and a recombinant protein of Chlamydia.

Once the antigen or antigenic determinant of the composition is chosen, at least one second attachment site may be added to the molecule in preparing to construct the organized and repetitive array associated with the non-natural molecular scaffold of the invention. Knowledge of what will constitute an appropriate second attachment site will be known to those skilled in the art. Representative examples of second attachment sites include, but are not limited to, the following: an antigen, an antibody or antibody fragment, biotin, avidin, streptavidin, a receptor, a receptor ligand, a ligand, a ligand-binding protein, an interacting leucine zipper polypeptide, an amino group, a chemical group reactive to an amino group; a carboxyl group, chemical group reactive to a carboxyl group, a sulfhydryl group, a chemical group reactive to a sulfhydryl group, or a combination thereof.

The association between the first and second attachment sites will be determined by the characteristics of the respective molecules selected but will comprise at least one non-peptide bond. Depending upon the combination of first and second attachment sites, the nature of the association may be covalent, ionic, hydrophobic, polar, or a combination thereof.

In one embodiment of the invention, the second attachment site may be the *FOS* leucine zipper protein domain or the *JUN* leucine zipper protein domain.

In a most specific embodiment of the invention, the second attachment site selected is the *FOS* leucine zipper protein domain, which associates specifically with the *JUN* leucine zipper protein domain of the non-natural molecular scaffold of the invention. The association of the *JUN* and *FOS* leucine zipper protein domains provides a basis for the formation of an organized and repetitive antigen or antigenic determinant array on the surface of the scaffold. The *FOS* leucine zipper protein domain may be fused in frame to the antigen or antigenic determinant of choice at either the amino terminus, carboxyl terminus or internally located in the protein if desired.



Several *FOS* fusion constructs are provided for exemplary purposes. Human growth hormone (Example 4), bee venom phospholipase A<sub>2</sub> (PLA) (Example 9), ovalbumin (Example 10) and HIV gp140 (Example 12).

In order to simplify the generation of *FOS* fusion constructs, several vectors are disclosed that provide options for antigen or antigenic determinant design and construction (*see* Example 6). The vectors pAV1-4 were designed for the expression of *FOS* fusion in *E. coli*; the vectors pAV5 and pAV6 were designed for the expression of *FOS* fusion proteins in eukaryotic cells. Properties of these vectors are briefly described:

1. pAV1: This vector was designed for the secretion of fusion proteins with *FOS* at the C-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) may be ligated into the *Stu*I/*Not*I sites of the vector.

2. pAV2: This vector was designed for the secretion of fusion proteins with *FOS* at the N-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) ligated into the *Not*I/*Eco*RV (or *Not*I/*Hind*III) sites of the vector.

3. pAV3: This vector was designed for the cytoplasmic production of fusion proteins with *FOS* at the C-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the *Eco*RV/*Not*I sites of the vector.

4. pAV4: This vector is designed for the cytoplasmic production of fusion proteins with *FOS* at the N-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the *Not*I/*Eco*RV (or *Not*I/*Hind*III) sites of the vector. The N-terminal methionine residue is proteolytically removed upon protein synthesis (Hirel *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8247-8251 (1989)).

5. pAV5: This vector was designed for the eukaryotic production of fusion proteins with *FOS* at the C-terminus. The gene of interest (g.o.i.) may be inserted between the sequences coding for the hGH signal sequence and the *FOS* domain by ligation into the *Eco*47III/*Not*I sites of the vector. Alternatively, a gene containing its own signal sequence may be fused to the *FOS* coding region by ligation into the *Stu*I/*Not*I sites.

6. pAV6: This vector was designed for the eukaryotic production of fusion proteins with *FOS* at the N-terminus. The gene of interest (g.o.i.) may be ligated into the NotI/StuI (or NotI/HindIII) sites of the vector.

As will be understood by those skilled in the art, the construction of a *FOS*-antigen or -antigenic determinant fusion protein may include the addition of certain genetic elements to facilitate production of the recombinant protein. Example 4 provides guidance for the addition of certain *E. coli* regulatory elements for translation, and Example 7 provides guidance for the addition of a eukaryotic signal sequence. Other genetic elements may be selected, depending on the specific needs of the practitioner.

The invention is also seen to include the production of the *FOS*-antigen or *FOS*-antigenic determinant fusion protein either in bacterial (Example 5) or eukaryotic cells (Example 8). The choice of which cell type in which to express the fusion protein is within the knowledge of the skilled artisan, depending on factors such as whether post-translational modifications are an important consideration in the design of the composition.

As noted previously, the invention discloses various methods for the construction of a *FOS*-antigen or *FOS*-antigenic determinant fusion protein through the use of the pAV vectors. In addition to enabling prokaryotic and eukaryotic expression, these vectors allow the practitioner to choose between – and C- terminal addition to the antigen of the *FOS* leucine zipper protein domain. Specific examples are provided wherein – and C- terminal *FOS* fusions are made to PLA (Example 9) and ovalbumin (Example 10). Example 11 demonstrates the purification of the PLA and ovalbumin *FOS* fusion proteins.

In a most specific embodiment, the invention is drawn to an antigen or antigenic determinant encoded by the HIV genome. More specifically, the HIV antigen is gp140. As provided for in Examples 11-15, HIV gp140 may be created with a *FOS* leucine zipper protein domain and the fusion protein synthesized and purified for attachment to the non-natural molecular scaffold of the invention. As

one skilled in the art would know, other HIV antigens or antigenic determinants may be used in the creation of a composition of the invention.

In a most specific embodiment of the invention, the second attachment site selected is a cysteine residue, which associates specifically with a lysine residue of the non-natural molecular scaffold of the invention. The chemical linkage of the lysine residue (Lys) and cysteine residue (Cys) provides a basis for the formation of an organized and repetitive antigen or antigenic determinant array on the surface of the scaffold. The cysteine residue may be engineered in frame to the antigen or antigenic determinant of choice at either the amino terminus, carboxyl terminus or internally located in the protein if desired. By way of example, PLA and HIV gp140 are provided with a cysteine residue for linkage to a lysine residue first attachment site.

### *C. Preparation of the AlphaVaccine Particles*

The invention provides novel compositions and methods for the construction of ordered and repetitive antigen arrays. As one of skill in the art would know, the conditions for the assembly of the ordered and repetitive antigen array depend to a large extent on the specific choice of the first attachment site of the non-natural scaffold and the specific choice of the second attachment site of the antigen or antigenic determinant. Thus, practitioner choice in the design of the composition (*i.e.*, selection of the first and second attachment sites, antigen and non-natural scaffold) will determine the specific conditions for the assembly of the AlphaVaccine particle (the ordered and repetitive antigen array and non-natural molecular scaffold combined). Information relating to assembly of the AlphaVaccine particle is well within the working knowledge of the practitioner, and numerous references exist to aid the practitioner (*e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc.

(1997); Celis, J., ed., CELL BIOLOGY, Academic Press. 2<sup>nd</sup> edition, (1998); Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988), all of which are incorporated herein by reference.

5           In a specific embodiment of the invention, the *JUN* and *FOS* leucine zipper protein domains are utilized for the first and second attachment sites of the invention, respectively. In the preparation of AlphaVaccine particles, antigen must be produced and purified under conditions to promote assembly of the ordered and repetitive antigen array onto the non-natural scaffold. In the  
10       particular *JUN/FOS* leucine zipper protein domain embodiment, the *FOS*-antigen or *FOS*-antigenic determinant should be treated with a reducing agent (*e.g.*, Dithiothreitol (DTT)) to reduce or eliminate the incidence of disulfide bond formation (Example 15).

15           For the preparation of the non-natural scaffold (*i.e.*, recombinant Sinbis virus) of the *JUN/FOS* leucine zipper protein domain embodiment, recombinant E2-*JUN* viral particles should be concentrated, neutralized and treated with reducing agent (*see* Example 16).

20           Assembly of the ordered and repetitive antigen array in the *JUN/FOS* embodiment is done in the presence of a redox shuffle. E2-*JUN* viral particles are combined with a 240 fold molar excess of *FOS*-antigen or *FOS*-antigenic determinant for 10 hours at 4°C. Subsequently, the AlphaVaccine particle is concentrated and purified by chromatography (Example 16).

25           In another embodiment of the invention, the coupling of the non-natural molecular scaffold to the antigen or antigenic determinant may be accomplished by chemical crosslinking. In a most preferred embodiment, the chemical agent  
30       is a hetero-bifunctional crosslinking agent such as  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (Tanimori *et al.*, *J. Pharm. Dyn.* 4:812 (1981); Fujiwara *et al.*, *J. Immunol. Meth.* 45:195 (1981), which contains (1) a succinimide group reactive with amino groups and (2) a maleimide group reactive with SH groups.. A heterologous protein or polypeptide of the first attachment

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5 site may be engineered to contain one or more lysine residues that will serve as a reactive moiety for the succinimide portion of the hetero-bifunctional crosslinking agent. Once chemically coupled to the first attachment sites of the non-natural molecular scaffold, the maleimide group of the hetero-bifunctional crosslinking agent will be available to react with the SH group of a cysteine residue on the antigen or antigenic determinant. Antigen or antigenic determinant preparation in this instance may require the engineering of a cysteine residue into the protein or polypeptide chosen as the second attachment site so that it may be reacted to the free maleimide function on the crosslinking agent bound to the non-natural molecular scaffold first attachment sites.

### 3. *Compositions, Vaccines, and the Administration Thereof, and Methods of Treatment*

15 In one embodiment, the invention provides vaccines for the prevention of infectious diseases in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines may be designed to treat infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc.

20 In another embodiment, the invention provides vaccines for the prevention of cancer in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines may be designed to treat all types of cancer: lymphomas, carcinomas, sarcomas, melanomas, etc.

25 In another embodiment of the invention, the compositions of the invention may be used in the design of vaccines for the treatment of allergies. Antibodies of the IgE isotype are important components in allergic reactions. Mast cells bind IgE antibodies on their surface and release histamines and other mediators of allergic response upon binding of specific antigen to the IgE molecules bound on

the mast cell surface. Inhibiting production of IgE antibodies, therefore, is a promising target to protect against allergies. This should be possible by attaining a desired T helper cell response. T helper cell responses can be divided into type 1 ( $T_H1$ ) and type 2 ( $T_H2$ ) T helper cell responses (Romagnani, *Immunol. Today* 18:263-266 (1997)).  $T_H1$  cells secrete interferon-gamma and other cytokines which trigger B cells to produce IgG1-3 antibodies. In contrast, a critical cytokine produced by  $T_H2$  cells is IL-4, which drives B cells to produce IgG4 and IgE. In many experimental systems, the development of  $T_H1$  and  $T_H2$  responses is mutually exclusive since  $T_H1$  cells suppress the induction of  $T_H2$  cells and *vice versa*. Thus, antigens that trigger a strong  $T_H1$  response simultaneously suppress the development of  $T_H2$  responses and hence the production of IgE antibodies. Interestingly, virtually all viruses induce a  $T_H1$  response in the host and fail to trigger the production of IgE antibodies (Coutelier *et al.*, *J. Exp. Med.* 165:64-69 (1987)). This isotype pattern is not restricted to live viruses but has also been observed for inactivated or recombinant viral particles (Lo-Man *et al.*, *Eur. J. Immunol.* 28:1401-1407 (1998)). Thus, by using the processes of the invention (e.g., AlphaVaccine Technology), viral particles can be decorated with various allergens and used for immunization. Due to the resulting "viral structure" of the allergen, a  $T_H1$  response will be elicited, "protective" IgG1-3 antibodies will be produced, and the production of IgE antibodies which cause allergic reactions will be prevented. Since the allergen is presented by viral particles which are recognized by a different set of helper T cells than the allergen itself, it is likely that the allergen-specific IgG1-3 antibodies will be induced even in allergic individuals harboring pre-existing  $T_H2$  cells specific for the allergen. The presence of high concentrations of IgG antibodies may prevent binding of allergens to mast cell bound IgE, thereby inhibiting the release of histamine. Thus, presence of IgG antibodies may protect from IgE mediated allergic reactions. Typical substances causing allergies include: grass, ragweed, birch or mountain cedar pollens, house dust, mites, animal danders, mold, insect venom or drugs (e.g. penicillin). Thus, immunization of individuals with allergen-

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decorated viral particles should be beneficial not only before but also after the onset of allergies.

5 As would be understood by one of ordinary skill in the art, when the compositions of the invention are administered to an individual, they may be in a composition which contains salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. Examples of materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including REMINGTON'S PHARMACEUTICAL SCIENCES (Osol, A, ed., Mack Publishing Co., (1980)).

10 The compositions of the invention are said to be "pharmacologically acceptable" if their administration can be tolerated by a recipient individual. Further, the compositions of the invention will be administered in a "therapeutically effective amount" (*i.e.*, an amount that produces a desired physiological effect).

15 The compositions of the present invention may be administered by various methods known in the art, but will normally be administered by injection, infusion, inhalation, oral administration, or other suitable physical methods. The compositions may alternatively be administered intramuscularly, intravenously, or subcutaneously. Components of compositions for administration include sterile aqueous (*e.g.*, physiological saline) or non-aqueous solutions and suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption.

20 In addition to vaccine technologies, other embodiments of the invention are drawn to methods of medical treatment for cancer and allergies.

25 All patents and publications referred to herein are expressly incorporated by reference.

### *Examples*

Enzymes and reagents used in the experiments that follow included: T4 DNA ligase obtained from New England Biolabs; Taq DNA Polymerase, QIAprep Spin Plasmid Kit, QIAGEN Plasmid Midi Kit, QiaExII Gel Extraction Kit, QIAquick PCR Purification Kit obtained from QIAGEN; QuickPrep Micro mRNA Purification Kit obtained from Pharmacia; SuperScript One-step RT PCR Kit, fetal calf serum (FCS), bacto-tryptone and yeast extract obtained from Gibco BRL; Oligonucleotides obtained from Microsynth (Switzerland); restriction endonucleases obtained from Boehringer Mannheim, New England Biolabs or MBI Fermentas; Pwo polymerase and dNTPs obtained from Boehringer Mannheim. HP-1 medium was obtained from Cell culture technologies (Glattbrugg, Switzerland). All standard chemicals were obtained from Fluka-Sigma-Aldrich, and all cell culture materials were obtained from TPP.

DNA manipulations were carried out using standard techniques. DNA was prepared according to manufacturer instruction either from a 2 ml bacterial culture using the QIAprep Spin Plasmid Kit or from a 50 ml culture using the QIAGEN Plasmid Midi Kit. For restriction enzyme digestion, DNA was incubated at least 2 hours with the appropriate restriction enzyme at a concentration of 5-10 units (U) enzyme per mg DNA under manufacturer recommended conditions (buffer and temperature). Digests with more than one enzyme were performed simultaneously if reaction conditions were appropriate for all enzymes, otherwise consecutively. DNA fragments isolated for further manipulations were separated by electrophoresis in a 0.7 to 1.5% agarose gel, excised from the gel and purified with the QiaExII Gel Extraction Kit according to the instructions provided by the manufacturer. For ligation of DNA fragments, 100 to 200 pg of purified vector DNA were incubated overnight with a threefold molar excess of the insert fragment at 16°C in the presence of 1 U T4 DNA ligase in the buffer provided by the manufacturer (total volume: 10-20 µl). An aliquot (0.1 to 0.5 µl) of the ligation reaction was used for transformation of



*E. coli* XL1-Blue (Stratagene). Transformation was done by electroporation using a Gene Pulser (BioRAD) and 0.1 cm Gene Pulser Cuvettes (BioRAD) at 200  $\Omega$ , 25  $\mu$ F, 1.7 kV. After electroporation, the cells were incubated with shaking for 1 h in 1 ml S.O.B. medium (Miller, 1972) before plating on selective S.O.B. agar.

5

**Example 1:**

***Insertion of the JUN amphiphatic helix domain within E2***

In the vector pTE5'2J (Hahn *et al.*, *Proc. Natl. Acad. Sci. USA* 89:2679-2683, (1992)), *Mlu*I and a *Bst*EII restriction enzyme sites were introduced between codons 71 (Gln) and 74 (Thr) of the structural protein E2 coding sequence, resulting in vector pTE5'2JBM. Introduction of these restriction enzymes sites was done by PCR using the following oligonucleotides:

10

Oligo 1:

E2ins*Bst*EII/*Bss*HII:

15

5'-ggggACGCGTGCAGCAggtaccaccgTTAAAGAAGGCACC-3' (SEQ ID NO:1)

Oligo 2:

E2ins*Mlu*I/*Stu*I:

5'-cgggtggtaccTGCTGCACGCGTTGCTTAAGCGACATGTAGCGG-3' (SEQ ID NO:2)

20

Oligo 3:

E2ins*Stu*I: 5'-CCATGAGGCCTACGATACCC-3' (SEQ ID NO:3)

Oligo4:

E2ins*Bss*HII: 5'-GGCACTCACGGCGCGCTTTACAGGC-3' (SEQ ID NO:4)

25

For the PCR reaction, 100 pmol of each oligo was used with 5 ng of the template DNA in a 100  $\mu$ l reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM  $MgSO_4$ . All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following manner and order: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 53°C (60 seconds), 72°C

30

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(80 seconds); and 25 cycles of 95°C (45 seconds), 57°C (60 seconds), 72°C (80 seconds).

5 The two PCR fragments were analyzed and purified by agarose gelelectrophoresis. Assembly PCR of the two PCR fragments using oligo 3 and 4 for amplification was carried out to obtain the final construct.

10 For the assembly PCR reaction, 100 pmol of each oligo was used with 2 ng of the purified PCR fragments in a 100 µl reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following manner and order: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 57°C (60 seconds), 72°C (90 seconds); and 25 cycles of 95°C (45 seconds), 59°C (60 seconds), 72°C (90 seconds).

15 The final PCR product was purified using Qia spin PCR columns (Qiagen) and digested in an appropriate buffer using 10 units each of BssHII and StuI restriction endonucleases for 12 hours at 37°C. The DNA fragments were gel-purified and ligated into BssHII/StuI digested and gel-purified pTE5'2J vector (Hahn *et al.*, *Proc. Natl. Acad. Sci. USA* 89:2679-2683). The correct insertion of  
20 the PCR product was first analyzed by BstEII and MluI restriction analysis and then by DNA sequencing of the PCR fragment.

The DNA sequence coding for the *JUN* amphiphatic helix domain was PCR-amplified from vector pJuFo (Cramer and Suter, *Gene* 137:69 (1993)) using the following oligonucleotides:

25 Oligo 5:  
*JUN*/BstEII:  
5'-CCTTCTTTAAcgggtggtaccTGCTGGCAACCAACGTGGTTCATGAC-3'  
(SEQ ID NO:5)

30 Oligo 6:  
MluI/*JUN*: 5'-AAGCATGCTGCacgcgtgTGCGGTGGTCGGATCGCCCGGC-3'  
(SEQ ID NO:6)

For the PCR reaction, 100 pmol of each oligo was used with 5 ng of the template DNA in a 100 µl reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following order and manner: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 60°C (30 seconds), 72°C (25 seconds); and 25 cycles of 95°C (45 seconds), 68°C (30 seconds), 72°C (20 seconds).

The final PCR product was gel-purified and ligated into EcoRV digested and gel-purified pBluescript II(KS<sup>-</sup>). From the resulting vector, the *JUN* sequence was isolated by cleavage with *MluI/BstEII* purified with QiaExII and ligated into vector pTE5'2JBM (previously cut with the same restriction enzymes) to obtain the vector pTE5'2J:E2*JUN*.

#### Example 2:

##### *Production of viral particles containing E2-JUN using the pCYTts system*

The structural proteins were PCR amplified using pTE5'2J:E2*JUN* as template and the oligonucleotides XbaIStruct (ctatcaTCTAGAATGAATAGAGGATTCTTTAAC) and StructBsp1201 (tcgaatGGGCCCTCATCTTCGTGTGCTAGTCAG). For the PCR 100 pmol of each oligo was used and 5 ng of the template DNA was used in the 100 µl reaction mixture, containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). The polymerase was added directly before starting the PCR reaction (starting point was 95°C). The temperature cycles were as follows: 95°C for 3 minutes, followed by 5 cycles of 92°C (30 seconds), 54°C (35 seconds), 72°C (270 seconds) and followed by 25 cycles of 92°C (30 seconds), 63°C (35 seconds), 72°C (270 seconds). The PCR product was gel purified and digested with the restriction enzymes XbaI/Bsp1201 and

ligated into vector pCYTts previously cleaved with the same enzymes (US Patent Application Appl. No. 60/079,562; Filed March 27,1998)

Twenty µg of pCYTtsE2:JUN were incubated with 30 U of ScaI in an appropriate buffer for at least 4 hours at 37°C. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linerized DNA. The restriction reaction was checked by agarose gel electrophoresis. For the transfection, 5.4 µg of linearized pCYTtsE2:JUN was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution were added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub> HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for 25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture in a 6-well plate was then replaced with the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

Stably transfected cells were selected and grown in selection medium (HP-1 medium, supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 hours growth period at 37°C. One dish of the cells was shifted to 30°C to induce the expression of the viral particles; the other dish was kept at 37°C.

The expression of viral particles was determined by Western blotting (Figure 1). Culture medium (0.5 ml) was methanol/chloroform precipitated, and the pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes

(Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1% bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1 g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-E1/E2 antibody (polyclonal serum) for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-rabbit IgG conjugate (0.1 µg/ml, Amersham Life Science, England). After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50 µl NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37 µl of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide).

The production of viral particles is shown in Figure 1. The Western Blot pattern showed that E2-JUN (lane 1) migrated to a higher molecular weight in SDS-PAGE compared to wild type E2 (lane 2) and the BHK21 host cell line did not show any background.

**Example 3:**  
***Production of viral particles containing E2-JUN***  
***using the pTE5 2JE2:JUN vector***

RNase-free vector (1.0 µg) was linearized by PvuI digestion. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscriptCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on a reducing agarose-gel.

*In vitro* transcribed mRNA (5 µg) was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression system, Invitrogen BV, Netherlands). After 10 hours incubation at 37°C, the FCS containing medium was exchanged by HP-1 medium without FCS, followed by

an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by Western blot analysis for production of viral particles exactly as described in Example 2.

The obtained result was identical to the one obtained with pCYTsE2:*JUN* as shown in Figure 2.

**Example 4:**

***Fusion of human growth hormone (hGH) to the FOS leucine zipper domain (OmpA signal sequence)***

The hGH gene without the human leader sequence was amplified from the original plasmid (ATCC 31389) by PCR. Oligo 7 with an internal XbaI site was designed for annealing at the 5' end of the hGH gene, and oligo 9 with an internal EcoRI site primed at the 3' end of the hGH gene. For the PCR reaction, 100 pmol of each oligo and 5 ng of the template DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>).

PCR cycling was performed in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 1 minute at 72°C.

The gel purified and isolated PCR product was used as a template for a second PCR reaction to introduce the ompA signal sequence and the Shine-Dalgarno sequence. For the PCR reaction, 100 pmol of oligo 8 and 9 and 1 ng of the template PCR fragment was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). The annealing temperature for the first five cycles was 55°C with an elongation time of 60 seconds at 72°C; another 25 cycles were performed with an annealing temperature of 65°C and an elongation time of 60 seconds at 72°C.

*Oligo 7:*

gggtctagattccaaccattcccttatccaggcttttgacaacgctatgctccgcgcccatcgtctgcaccagct  
ggcctttgacacc (SEQ ID NO:7)

*Oligo 8:*

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gggtctagaaggaggtaaaaacgatgaaaagacagctatcgcgattgcagtggcactggctggttcgctac  
cgtagcgcaggccttcccaaccattccctatcc (SEQ ID NO:8)

*Oligo 9:*

cccgaattcctagaagccacagctgccctcc (SEQ ID NO:9)

5           The resulting recombinant hGH gene was subcloned into pBluescript via  
XbaI/EcoRI. The correct sequence of both strands was confirmed by DNA  
sequencing.

          The DNA sequence coding for the *FOS* amphipathic helix domain was  
PCR-amplified from vector pJuFo (*Cramer & Suter Gene 137:69 (1993)*) using  
10       the oligonucleotides:

omp-*FOS*:

5'- ccTGCGGTGGTCTGACCGACACCC-3' (SEQ ID NO:10)

*FOS*-hgh:

5'- ccgcggaagagccaccGCAACCACCGTGTGCCGCCAGGATG-3' (SEQ ID  
15       NO:11)

          For the PCR reaction, 100 pmol of each oligo and 5 ng of the template  
DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase,  
0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). The temperature cycles were as follows:

20       95°C for 2 minutes, followed by 5 cycles of 95°C (45 seconds), 60°C  
(30 seconds), 72°C (25 seconds) and followed by 25 cycles of 95°C (45 seconds),  
68°C (30 seconds), 72°C (20 seconds).

          The PCR product was purified, isolated and cloned into the StuI digested  
pBluescript-ompA-hGH. The hybrid gene was then cloned into the pKK223-3  
Plasmid (Pharmacia).

25

#### *Example 5:*

#### *Bacterial expression of FOS-hGH*

          The ompA-*FOS*-hGH in pkk223-3 was expressed under the control of the  
inducible IPTG-dependend promoter using JM101 as *E. coli* host strain.  
Expression was performed in shaker flask. Cells were induced with 1 mM IPTG

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(final concentration) at an OD600 of 0.5. Expression was continued for 10 hours at 37°C. Cells were harvested by centrifugation at 3600 at 10°C for 15 min. The cell pellet was frozen (-20°C or liq. N<sub>2</sub>) and stored for 16 hours. The pellet was then thawed at 4°C and resuspended in 10 ml 10 mM Tris-HCl, pH 7.4 containing 600 mM sucrose. After stirring for 15 min at 4°C, periplasmic proteins were released by an osmotic shock procedure. Chilled (4°C) deionized H<sub>2</sub>O was added, and the suspension was stirred for 30 min at 4°C. The sludge was diluted, resuspended, and lysozyme was added to degrade the cell wall of the bacteria. The cells and the periplasmic fraction spheroplasts were separated by centrifugation for 20 min at 11000 g at 4°C. The *FOS*-hGH-containing supernatant was analyzed by reducing and non-reducing SDS-Page and Dot Blot. Dot Blot was carried out as described in Example 8, using an anti-hGH antibody (Sigma) as the first antibody and an alkaline phosphatase (AP)-anti-mouse antibody conjugate as the second antibody.

Full length, correctly processed *FOS*-hGH could be detected under reducing and non-reducing conditions. Part of *FOS*-hGH was bound to other, non-identified proteins due to the free cysteines present in the *FOS* amphipathic helix. However, more than 50% of expressed *FOS*-hGH occurred in its native monomeric conformation ( Figure 3).

Purified *FOS*-hGH will be used to perform first doping experiments with *JUN* containing viral particles.

#### **Example 6:**

##### ***Construction of the pAV vector series for expression of FOS fusion proteins***

A versatile vector system was constructed that allowed either cytoplasmic production or secretion of – or C-terminal *FOS* fusion proteins in *E. coli* or production of – or C-terminal *FOS* fusion proteins in eukaryotic cells. The vectors pAV1 - pAV4 which was designed for production of *FOS* fusion proteins in *E. coli*, encompasses the DNA cassettes listed below, which contain the following genetic elements arranged in different orders: (a) a strong ribosome binding site and 5'-untranslated region derived from the *E. coli* ompA gene



-47-

(aggaggtaaaaaacg) (SEQ ID NO:13); (b) a sequence encoding the signal peptide of *E. coli* outer membrane protein OmpA (MKKTAIAIAVALAGFATVAQA) (SEQ ID NO:14); (c) a sequence coding for the *FOS* dimerization domain flanked on both sides by two glycine residues and a cystein residue

5 (CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC) (SEQ ID NO:15); and (d) a region encoding a short peptidic linker (AAASGG (SEQ ID NO:16) or GGSAAA (SEQ ID NO:17)) connecting the protein of interest to the *FOS* dimerization domain. Relevant coding regions are given in upper case letters. The arrangement of restriction cleavage sites allows easy

10 construction of *FOS* fusion genes with or without a signal sequence. The cassettes are cloned into the EcoRI/HindIII restriction sites of expression vector pKK223-3 (Pharmacia) for expression of the fusion genes under control of the strong tac promotor.

pAV1

15 This vector was designed for the secretion of fusion proteins with *FOS* at the C-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) may be ligated into the StuI/NotI sites of the vector.

20 EcoRI 31/11  
gaa ttc agg agg taa aaa acg ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT  
M K K T A I A I A V A L A

61/21 StuI NotI  
GGT TTC GCT ACC GTA GCG CAG GCC tgg gtg ggg GCG GCC GCT TCT GGT GGT TGC GGT GGT  
G F A T V A Q A (goi) A A A S G G C G G

25 121/41 151/51  
CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA  
L T D T L Q A E T D Q V E D E K S A L Q

181/61 211/71  
ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC  
T E I A N L L K E K E K L E F I L A A H

30 241/81 HindIII  
GGT GGT TGC taa gct t (SEQ ID NO:18)  
G G C \* A (SEQ ID NO:19)

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pAV2

This vector was designed for the secretion of fusion proteins with *FOS* at the N-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector.

```

5      EcoRI                               31/11
      gaa ttc agg agg taa aaa acg ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT
                                   M K K T A I A I A V A L A

      61/21                               StuI                               91/31
10     GGT TTC GCT ACC GTA GCG CAG GCC TGC GGT GGT CTG ACC GAC ACC CTG CAG GCG GAA ACC
      G F A T V A Q A C G G L T D T L Q A E T

      121/41                               151/51
      GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA ACC GAA ATC GCG AAC CTG CTG AAA GAA
      D Q V E D E K S A L Q T E I A N L L K E

15     181/61                               211/71                               NotI
      AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC GGT GGT TGC GGT GGT TCT GCG GCC GCT
      K E K L E F I L A A H G G C G G S A A A

      241/81      EcoRV      HindIII
      999 tgt ggg gat atc aag ctt      (SEQ ID NO:20)
      (goi)                               (SEQ ID NO:21)

```

pAV3

This vector was designed for the cytoplasmic production of fusion proteins with *FOS* at the C-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the EcoRV/NotI sites of the vector.

```

25     EcoRI                               EcoRV                               NotI
      gaa ttc agg agg taa aaa gat atc ggg tgt ggg GCG GCC GCT TCT GGT GGT TGC GGT GGT
                                   (goi)      A A A S G G C G G

      61/21                               91/31
      CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA
      L T D T L Q A E T D Q V E D E K S A L Q

30     121/41                               151/51
      ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC
      T E I A N L L K E K E K L E F I L A A H

      181/61      HindIII
35     GGT GGT TGC taa gct t      (SEQ ID NO:22)
      G G C *                               (SEQ ID NO:23)

```

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pAV4

This vector is designed for the cytoplasmic production of fusion proteins with *FOS* at the N-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector. The N-terminal methionine residue is proteolytically removed upon protein synthesis (Hirel *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8247-8251 (1989)).

```

EcoRI                               31/11
gaa ttc agg agg taa aaa acg ATG GCT TGC GGT GGT CTG ACC GAC ACC CTG CAG GCG GAA
E  F  R  R  *  K  T  M  A  C  G  G  L  T  D  T  L  Q  A  E

10  61/21                               91/31
ACC GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA ACC GAA ATC GCG AAC CTG CTG AAA
T  D  Q  V  E  D  E  K  S  A  L  Q  T  E  I  A  N  L  L  K

15  121/41                               151/51                               NotI
GAA AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC GGT GGT TGC GGT GGT TCT GCG GCC
E  K  E  K  L  E  F  I  L  A  A  H  G  G  C  G  G  S  A  A

181/61                               EcoRV   HindIII
GCT ggg tgt ggg gat atc aag ctt (SEQ ID NO:24)
A      (goi)                      (SEQ ID NO:25)

```

The vectors pAV5 and pAV6, which are designed for eukaryotic production of *FOS* fusion proteins, encompasses the following genetic elements arranged in different orders: (a) a region coding for the leader peptide of human growth hormone (MATGSRTSLLLAFLGLCLPWLQEGSA) (SEQ ID NO:26); (b) a sequence coding for the *FOS* dimerization domain flanked on both sides by two glycine residues and a cysteine residue (CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC) (SEQ ID NO:15); and (c) a region encoding a short peptidic linker (AAASGG (SEQ ID NO:16) or GGSAAA (SEQ ID NO:17)) connecting the protein of interest to the *FOS* dimerization domain. Relevant coding regions are given in upper case letters. The arrangement of restriction cleavage sites allows easy construction of *FOS* fusion genes. The cassettes are cloned into the EcoRI/HindIII restriction sites of the expression vector pMPSVEH (Artelt *et al.*, *Gene* 68:213-219 (1988)).

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pAV5

This vector is designed for the eukaryotic production of fusion proteins with *FOS* at the C-terminus. The gene of interest (g.o.i.) may be inserted between the sequences coding for the hGH signal sequence and the *FOS* domain by ligation into the Eco47III/NotI sites of the vector. Alternatively, a gene containing its own signal sequence may be fused to the *FOS* coding region by ligation into the StuI/NotI sites.

```

10      EcoRI   StuI                               31/11
      gaa ttc agg cct ATG GCT ACA GGC TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG CTC
                               M  A  T  G  S  R  T  S  L  L  L  A  F  G  L  L

      61/21                               Eco47III           NotI
      TGC CTG CCC TGG CTT CAA GAG GGC AGC GCT ggg tgt ggg GCG GCC GCT TCT GGT GGT TGC
      C  L  P  W  L  Q  E  G  S  A  (goi)  A  A  A  S  G  G  C

15      121/41                               151/51
      GGT GGT CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC GAA AAA TCC GCG
      G  G  L  T  D  T  L  Q  A  E  T  D  Q  V  E  D  E  K  S  A

      181/61                               211/71
      CTG CAA ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG TTC ATC CTG GCG
      L  Q  T  E  I  A  N  L  L  K  E  K  E  K  L  E  F  I  L  A

20      241/81                               HindIII
      GCA CAC GGT GGT TGC taa gct t (SEQ ID NO:27)
      A  H  G  G  C  * (SEQ ID NO:28)

```

pAV6

This vector is designed for the eukaryotic production of fusion proteins with *FOS* at the N-terminus. The gene of interest (g.o.i.) may be ligated into the NotI/StuI (or NotI/HindIII) sites of the vector.

```

30      EcoRI                               31/11
      gaa ttc ATG GCT ACA GGC TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG CTC TGC CTG
                               M  A  T  G  S  R  T  S  L  L  L  A  F  G  L  L  C  L

      61/21                               Eco47III           91/31
      CCC TGG CTT CAA GAG GGC AGC GCT TGC GGT GGT CTG ACC GAC ACC CTG CAG GCG GAA ACC
      P  W  L  Q  E  G  S  A  C  G  G  L  T  D  T  L  Q  A  E  T

35      121/41                               151/51
      GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA ACC GAA ATC GCG AAC CTG CTG AAA GAA
      D  Q  V  E  D  E  K  S  A  L  Q  T  E  I  A  N  L  L  K  E

```

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181/61 211/71 NotI

AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC GGT GGT TGC GGT GGT TCT GCG GCC GCT

K E K L E F I L A A H G G C G G S A A A

241/81 StuI HindIII

ggg tgt ggg agg cct aaq ctt (SEQ ID NO:29)

(goi) (SEQ ID NO:30)

### Construction of expression vectors pAV1 - pAV6

The following oligonucleotides have been synthesized for construction of expression vectors pAV1 - pAV6:

10 *FOS-FOR1:*  
CCTGGGTGGGGGCGGCCGCTTCTGGTGGTTGCGGTGGTCTGACC (SEQ  
ID NO:31);  
*FOS-FOR2:*  
GGTGGGAATTCAGGAGGTAAAAAGATATCGGGTGTGGGGCGGCC  
15 (SEQ ID NO:32);  
*FOS-FOR3:*  
GGTGGGAATTCAGGAGGTAAAAACGATGGCTTGCGGTGGTCTGACC  
(SEQ ID NO:33);  
*FOS-FOR4:*  
20 GCTTGCGGTGGTCTGACC (SEQ ID NO:34);  
*FOS-REV1:*  
CCACCAAGCTTAGCAACCACCGTGTGC (SEQ ID NO:35);  
*FOS-REV2:*  
CCACCAAGCTTGATATCCCCACACCCAGCGGCCGCAGAACCACCGC  
25 AACCACCG (SEQ ID NO:36);  
*FOS-REV3:*  
CCACCAAGCTTAGGCCTCCCACACCCAGCGGC (SEQ ID NO:37);  
*OmpA-FOR1:*  
GGTGGGAATTCAGGAGGTAAAAACGATG (SEQ ID NO:38);  
30 *hGH-FOR1:*

GGTGGGAATTCAGGCCTATGGCTACAGGCTCC (SEQ ID NO:39); and  
hGH-FOR2:

GGTGGGAATTCATGGCTACAGGCTCCC (SEQ ID NO:40).

5 For the construction of vector pAV2, the regions coding for the OmpA  
signal sequence and the *FOS* domain were amplified from the ompA-*FOS*-hGH  
fusion gene in vector pKK223-3 (*see* Example 5) using the primer pair  
OmpA-FOR1/ *FOS*-REV2. The PCR product was digested with EcoRI/HindIII  
and ligated into the same sites of vector pKK223-3 (Pharmacia).

10 For the construction of vector pAV1, the *FOS* coding region was  
amplified from the ompA-*FOS*-hGH fusion gene in vector pKK223-3 (*see*  
Example 5) using the primer pair *FOS*-FOR1/ *FOS*-REV1. The PCR product was  
digested with HindIII and ligated into StuI/HindIII digested vector pAV2.

15 For the construction of vector pAV3, the region coding for the *FOS*  
domain was amplified from vector pAV1 using the primer pair  
*FOS*-FOR2/*FOS*-REV1. The PCR product was digested with EcoRI/HindIII and  
ligated into the same sites of the vector pKK223-3 (Pharmacia).

20 For the construction of vector pAV4, the region coding for the *FOS*  
domain was amplified from the ompA-*FOS*-hGH fusion gene in vector  
pKK223-3 (*see* Example 5) using the primer pair *FOS*-FOR3/*FOS*-REV2. The  
PCR product was digested with EcoRI/HindIII and ligated into the same sites of  
the vector pKK223-3 (Pharmacia).

25 For the construction of vector pAV5, the region coding for the hGH signal  
sequence is amplified from the hGH-*FOS*-hGH fusion gene in vector pSINrep5  
(*see* Example 7) using the primer pair hGH-FOR1/hGHREV1. The PCR product  
is digested with EcoRI/NotI and ligated into the same sites of the vector pAV1.  
The resulting cassette encoding the hGH signal sequence and the *FOS* domain is  
then isolated by EcoRI/HindIII digestion and cloned into vector pMPSVEH  
(Artelt *et al.*, *Gene* 68:213-219 (1988)) digested with the same enzymes.

For the construction of vector pAV6, the *FOS* coding region is amplified from vector pAV2 using the primer pair *FOS*-FOR4/*FOS*REV3. The PCR product is digested with HindIII and cloned into Eco47III/HindIII cleaved vector pAV5. The entire cassette encoding the hGH signal sequence and the *FOS* domain is then reamplified from the resulting vector using the primer pair hGH-FOR2/*FOS*REV3, cleaved with EcoRI/HindIII and ligated into vector pMPSVEH (Artelt *et al.*, *Gene* 68:213-219 (1988)) cleaved with the same enzymes.

**Example 7:**

***Construction of FOS-hGH with human (hGH) signal sequence***

For eukaryotic expression of the *FOS*-hGH fusion protein, the OmpA-*FOS*-hGH fusion gene was isolated from pBluescript::OmpA-*FOS*-hGH (see Example 4) by digestion with XbaI/Bsp120I and cloned into vector pSINrep5 (Invitrogen) cleaved with the same enzymes. The hGH signal sequence was synthesized by PCR (reaction mix: 50 pmol of each primer, dATP, dGTP, dTTP, dCTP (200  $\mu$ M each), 2.5 U Taq DNA polymerase (Qiagen), 50  $\mu$ l total volume in the buffer supplied by the manufacturer; amplification: 92°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 30 cycles) using the overlapping oligonucleotides Sig-hGH-FOR

(GGGTCTAGAATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTT TTGGCCTGCTCTG) (SEQ ID NO:41) and Sig-hGH-REV

(CGCAGGCCTCGGCACTGCCCTCTTGAAGCCAGGGCAGGCAGAGCA GGCCAAAAGCCAG) (SEQ ID NO:42). The PCR product was purified using the QiaExII Kit, digested with StuI/XbaI and ligated into vector pSINrep5::OmpA-*FOS*-hGH cleaved with the same enzymes.

**Example 8:**  
***Eukaryotic expression of FOS-hGH***

5 RNase-free vector (1.0 µg) (pSINrep5::OmpA-FOS-hGH) and 1.0 µg of DHEB (Bredenbeek *et al.*, *J. Virol.* 67:6439-6446 (1993)) were linearized by ScaI restriction digest. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscripCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on reducing agarose-gel.

10 *In vitro*, transcribed mRNA 5 µg was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression system, Invitrogen BV, Netherlands). After 10 hours incubation at 37°C the FCS containing medium was exchanged by HP-1 medium without FCS, followed by an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by dot-blot analysis for production of FOS-hgh.

15 Culture media (2.5 µl) was spotted on a nitrocellulose membrane and dried for 10 minutes at room temperature. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with 2 µg rabbit anti-human hGH antibody (Sigma) in 10 ml TBS-T (TBS with 0.05% Tween20) for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T and incubated for 1 hour with alkaline phosphatase conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:5000 in TBS-T. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the blot was developed by AP staining as described in Example 2. Results are shown in Figure 3.

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**Example 9:**  
**Construction of FOS-PLA (N- and C-terminal)**

The following gene is constructed by chemical gene synthesis coding for a catalytically inactive variant (Förster *et al.*, *J. Allergy Clin. Immunol.* 95: 1229-1235 (1995)) of bee venom phospholipase A<sub>2</sub> (PLA).

```

1/1                               31/11
ATC ATC TAC CCA GGT ACT CTG TGG TGT GGT CAC GGC AAC AAA TCT TCT GGT CCG AAC GAA
I  I  Y  P  G  T  L  W  C  G  H  G  N  K  S  S  G  P  N  E

61/21                               91/31
CTC GGC CGC TTT AAA CAC ACC GAC GCA TGC TGT CGC ACC CAG GAC ATG TGT CCG GAC GTC
L  G  R  F  K  H  T  D  A  C  C  R  T  Q  D  M  C  P  D  V

121/41                              151/51
ATG TCT GCT GGT GAA TCT AAA CAC GGG TTA ACT AAC ACC GCT TCT CAC ACG CGT CTC AGC
M  S  A  G  E  S  K  H  G  L  T  N  T  A  S  H  T  R  L  S

181/61                              211/71
TGC GAC TGC GAC GAC AAA TTC TAC GAC TGC CTT AAG AAC TCC GCC GAT ACC ATC TCT TCT
C  D  C  D  D  K  F  Y  D  C  L  K  N  S  A  D  T  I  S  S

241/81                              271/91
TAC TTC GTT GGT AAA ATG TAT TTC AAC CTG ATC GAT ACC AAA TGT TAC AAA CTG GAA CAC
Y  F  V  G  K  M  Y  F  N  L  I  D  T  K  C  Y  K  L  E  H

301/101                             331/111
CCG GTA ACC GGC TGC GGC GAA CGT ACC GAA GGT CGC TGC CTG CAC TAC ACC GTT GAC AAA
P  V  T  G  C  G  E  R  T  E  G  R  C  L  H  Y  T  V  D  K

361/121                             391/131
TCT AAA CCG AAA GTT TAC CAG TGG TTC GAC CTG CGC AAA TAC      (SEQ ID NO:43)
S  K  P  K  V  Y  Q  W  F  D  L  R  K  Y      (SEQ ID NO:44)

```

For fusion of PLA to the N-terminus of the *FOS* dimerization domain, the region is amplified using the oligonucleotides PLA-FOR1 (CCATCATCTACCCAGGTAC) (SEQ ID NO:45) and PLA-REV1 (CCCACACCCAGCGGCCGCGTATTTGCGCAGGTCG) (SEQ ID NO:46). The PCR product is cleaved with NotI and ligated into vector pAV1 previously cleaved with the restriction enzymes StuI/NotI. For fusion of PLA to the C-terminus of the *FOS* dimerization domain, the region is amplified using the oligonucleotides PLA-FOR2 (CGGTGGTTCTGCGGCCGCTATCATCTACCCAGGTAC) (SEQ ID NO:47) and PLA-REV2 (TTAGTATTTGCGCAGGTCG) (SEQ ID NO:47). The PCR

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product is cleaved with NotI and ligated into vector pAV2 previously cleaved with the restriction enzymes NotI/EcoRV.

**Example 10:**

***Construction of FOS-Ovalbumin fusion gene (N- and C-terminal)***

5 For cloning of the ovalbumin coding sequence, mRNA from chicken oviduct tissue is prepared using the QuickPrep™ Micro mRNA Purification Kit (Pharmacia) according to manufacturer instructions. Using the SuperScript™ One-step RT PCR Kit (Gibco BRL), a cDNA encoding the mature part of ovalbumin (corresponding to nucleotides 68-1222 of the mRNA (McReynolds *et*  
10 *al.*, *Nature* 273:723-728 (1978)) is synthesized using the primers Ova-FOR1 (CCGGCTCCATCGGTGCAG) (SEQ ID NO:49) and Ova-REV1 (ACCACCAGAAGCGGCCGCAGGGGAAACACATCTGCC) (SEQ ID NO:50). The PCR product is digested with NotI and cloned into StuI/NotI digested vector pAV1 for expression of the fusion protein with the *FOS*  
15 dimerization domain at the C terminus. For production of a fusion protein with the *FOS* dimerization domain at the N terminus, the Ovalbumin coding region is amplified from the constructed vector (pAV1::Ova) using the primers Ova-FOR2 (CGGTGGTTCTGCGGCCGCTGGCTCCATCGGTGCAG) (SEQ ID NO:51) and Ova-REV2 (TTAAGGGGAAACACATCTGCC) (SEQ ID NO:52). The  
20 PCR product is digested with NotI and cloned into the NotI/EcoRV digested vector pAV2. Cloned fragments are verified by DNA sequence analysis.

**Example 11**

***Production and purification of FOS-PLA and FOS ovalbumin fusion proteins***

25 For cytoplasmic production of *FOS* fusion proteins, an appropriate *E. coli* strain was transformed with the vectors pAV3::PLA, pAV4::PLA, pAV3::Ova or pAV4::Ova. The culture was incubated in rich medium in the presence of ampicillin at 37°C with shaking. At an optical density (550nm) of 1, 1 mM IPTG was added and incubation was continued for another 5 hours. The cells were

harvested by centrifugation, resuspended in an appropriate buffer (e.g. tris-HCl, pH 7.2, 150 mM NaCl) containing DNase, RNase and lysozyme, and disrupted by passage through a french pressure cell. After centrifugation (Sorvall RC-5C, SS34 rotor, 15000 rpm, 10 min, 4°C), the pellet was resuspended in 25 ml inclusion body wash buffer (20 mM tris-HCl, 23% sucrose, 0.5% Triton X-100, 1 mM EDTA, pH8) at 4°C and recentrifuged as described above. This procedure was repeated until the supernatant after centrifugation was essentially clear. Inclusion bodies were resuspended in 20 ml solubilization buffer (5.5 M guanidinium hydrochloride, 25 mM tris-HCl, pH 7.5) at room temperature and insoluble material was removed by centrifugation and subsequent passage of the supernatant through a sterile filter (0.45 µm). The protein solution was kept at 4°C for at least 10 hours in the presence of 10 mM EDTA and 100 mM DTT and then dialyzed three times against 10 volumes of 5.5 M guanidinium hydrochloride, 25 mM tris-HCl, 10 mM EDTA, pH 6. The solution was dialyzed twice against 5 liters of 2 M urea, 4 mM EDTA, 0.1 M NH<sub>4</sub>Cl, 20 mM sodium borate (pH 8.3) in the presence of an appropriate redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine). The refolded protein was then applied to an ion exchange chromatography. The protein was stored in an appropriate buffer with a pH above 7 in the presence of 2-10 mM DTT to keep the cysteine residues flanking the FOS domain in a reduced form. Prior to coupling of the protein with the alphavirus particles, DTT was removed by passage of the protein solution through a Sephadex G-25 gel filtration column.

**Example 12:**  
**Constructions of gp140-FOS**

The gp140 gene (Swiss-Prot:P03375) without the internal protease cleavage site was amplified by PCR from the original plasmid pAbT4674 (ATCC 40829) containing the full length gp160 gene using the following oligonucleotides:

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HIV-1:

5'-ACTAGTCTAGAatgagagtgaaggagaaatc-3' (SEQ ID NO:53);

HIV-end:

5'-TAGCATGCTAGCACCGAAttatctaattccaataattcttg-3' (SEQ ID NO:54);

5 HIV-Cleav:

5'-gtagcaccaccaaggcaaagCTGAAAGCTACCCAGCTCGAGAAACTGgca-3'  
(SEQ ID NO:55); and

HIV-Cleav2:

10 5'-caaagctcctattccactgcCAGTTTCTCGAGCTGGGTAGCTTTCAG-3' (SEQ ID  
NO:56).

For PCR I, 100 pmol of oligo HIV-1 and HIV-Cleav2 and 5 ng of the template DNA were used in the 75  $\mu$ l reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 2 minutes at 72°C.

15

For PCR II, 100 pmol of oligo HIV-end and HIV-Cleav and 5 ng of the template DNA were used in the 75  $\mu$ l reaction mixture, (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 50 seconds at 72°C.

20

Both PCR fragments were purified, isolated and used in an assembly PCR reaction. For the assembly PCR reaction, 100 pmol of oligo HIV-1 and HIV-end and 2 ng of each PCR fragment (PCR I and PCR II) were used in the 75  $\mu$ l (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 2.5 minutes at 72°C. The assembly PCR product was digested XbaI and NheI. The *FOS* amphiphatic helix was fused in frame to the C-terminal end of gp-140.

25

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The DNA sequence coding for the *FOS* amphiphatic helix domain was PCR-amplified from vector pJuFo (Cramer & Suter *Gene* 137: 69 (1993)) using the oligonucleotides:

*FOS*-HIV:

5'-ttcgggtgctagcgggtggcTGCGGTGGTCTGACCGAC-3' (SEQ ID NO:57); and

*FOS*-Apa:

5'-gatgctggggccctaaccGCAACCACCGTGTGCCGCC-3' (SEQ ID NO:58).

For the PCR reaction, 100 pmol of each oligo and 5 ng of the template DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). Temperature cycling was done as follows: 95°C for 2 minutes, followed by 5 cycles of 95°C (45 seconds), 60°C (30 seconds), 72°C (25 seconds) and followed by 25 cycles of 95°C (45 seconds), 68°C (30 seconds), 72°C (20 seconds). The obtained PCR fragment was digested with NheI and Bsp120L.

The final expression vector for GP140-*FOS* was obtained in a 3 fragment ligation of both PCR fragments into pSinRep5. The resultant vector pSinRep5-GP140-*FOS* was evaluated by restriction analysis and DNA sequencing.

GP140-*FOS* was also cloned into pCYTts via XbaI and Bsp120L to obtain a stable, inducible GP140-*FOS* expressing cell line.

#### **Example 13:**

##### ***Expression of GP140FOS using pSinRep5-GP140FOS***

RNAse-free vector (1.0 µg) (pSinRep5-GP140-*FOS*) and 1.0 µg of DHEB (Bredenbeek *et al.*, *J. Virol.* 67:6439-6446 (1993)) were linearized by restriction digestion. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscipCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on a reducing agarose-gel.

*In vitro* transcribed mRNA (5 µg) was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression System,

Invitrogen BV, Netherlands). After 10 hours incubation at 37°C, the FCS containing medium was exchanged by HP-1 medium without FCS, followed by an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by Western blot analysis for production of soluble GP140-*FOS* exactly as described in Example 2.

**Example 14:**  
***Expression of GP140FOS using pCYTts-GP140FOS***

pCYT-GP140-*FOS* 20 µg was linearized by restriction digestion. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linearized DNA. The restriction digestion was evaluated by agarose gel electrophoresis. For the transfection, 5.4 µg of linearized pCYTtsGP140-*FOS* was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution was added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub> HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for 25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture (6-well plate) was then replaced by the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

Stably transfected cells were selected and grown in selection medium (HP-1 medium supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 h growth period at 37°C. One dish of the cells was shifted to 30°C to induce the expression of soluble GP140-*FOS*. The other dish was kept at 37°C.

The expression of soluble GP140-*FOS* was determined by Western blot analysis. Culture media (0.5 ml) was methanol/chloroform precipitated, and the pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to a 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-GP140 or GP-160 antibody for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-mouse/rabbit/monkey/human IgG conjugate. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50 µl NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37 µl of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide).

#### ***Example 15:***

##### ***Production and purification of GP140FOS***

An anti-gp120 antibody was covalently coupled to a NHS/EDC activated dextran and packed into a chromatography column. The supernatant, containing GP140FOS is loaded onto the column and after sufficient washing, GP140FOS was eluted using 0.1 M HCl. The eluate was directly neutralized during collection using 1 M Tris pH 7.2 in the collection tubes.

Disulfide bond formation might occur during purification, therefore the collected sample is treated with 10 mM DTT in 10 mM Tris pH 7.5 for 2 hours at 25°C.

DTT is removed by subsequent dialysis against 10 mM Mes; 80 mM NaCl pH 6.0. Finally GP140FOS is mixed with alphavirus particles containing the JUN leucine zipper in E2 as described in Example 16.

**Example 16:**

### *Preparation of the AlphaVaccine Particles*

Viral particles (*see* Examples 2 and 3) were concentrated using Millipore Ultrafree Centrifugal Filter Devices with a molecular weight cut-off of 100 kD according to the protocol supplied by the manufacturer. Alternatively, viral particles were concentrated by sucrose gradient centrifugation as described in the instruction manual of the Sindbis Expression System (Invitrogen, San Diego, California). The pH of the virus suspension was adjusted to 7.5 and viral particles were incubated in the presence of 2-10 mM DTT for several hours. Viral particles were purified from contaminating protein on a Sephacryl S-300 column (Pharmacia) (viral particles elute with the void volume) in an appropriate buffer.

Purified virus particles were incubated with at least 240 fold molar excess of FOS-antigen fusion protein in an appropriate buffer (pH 7.5-8.5) in the presence of a redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine) for at least 10 hours at 4°C. After concentration of the particles using a Millipore Ultrafree Centrifugal Filter Device with a molecular weight cut-off of 100 kD, the mixture was passed through a Sephacryl S-300 gel filtration column (Pharmacia). Viral particles were eluted with the void volume.

### Example 17

### *Fusion of JUN amphipathic helix to the amino terminus of HBcAg(1-144)*

25 The JUN helix was fused to the amino terminus of the HBcAg amino acid sequence 1 to 144 (JUN-HBcAg construct). For construction of the JUN-HBcAg DNA sequence, the sequences encoding the JUN helix and HBcAg(1 -144) were amplified separately by PCR. The JUN sequence was amplified from the pJuFo



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plasmid using primers EcoRI-JUN(s) and JUN-SacII(as). The EcoRI-JUN(s) primer introduced an EcoRI site followed by a start ATG codon. The JUN-SacII(as) primer introduced a linker encoding the amino acid sequence GAAGS. The HBcAg (1-144) sequence was amplified from the pEco63 plasmid (obtained from ATCC No. 31518) using primers JUN-HBcAg(s) and HBcAg(1-144)Hind(as). JUN-HBcAg(s) contained a sequence corresponding to the 3' end of the sequence encoding the JUN helix followed by a sequence encoding the GAAGS linker and the 5' end of the HBcAg sequence. HBcAg(1-144)Hind(as) introduces a stop codon and a HindIII site after codon 144 of the HBcAg gene. For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; and 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

***Primer sequences:***

EcoRI-JUN(s):

(5'-CCGGAATTCATGTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:61);

JUN-SacII(as):

(5'-GTCGCTACCCGCGGCTCCGCAACCAACGTGGTTCATGAC-3') (SEQ ID NO:62);

JUN-HBcAg(s):

(5'-GTTGGTTGCGGAGCCGCGGTAGCGACATTGACCCTTATAAAGAATTTGG-3') (SEQ ID NO:63);

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HBcAg(1-144)Hind(as):

(5'-CGCGTCCCAAGCTTCTACGGAAGCGTTGATAGGATAGG-3') (SEQ ID NO:64).

5 Fusion of the two PCR fragments was performed by PCR using primers  
EcoRI-JUN(s) and HBcAg(1-144)Hind(as). 100 pmol of each oligo was used with  
100ng of the purified PCR fragments in a 50 µl reaction mixture containing 2  
units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling  
conditions were: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C  
(1 minute), 72°C (2 minutes). The final PCR product was analyzed by agarose  
10 gel electrophoresis, purified and digested for 16 hours in an appropriate buffer  
with EcoRI and HindIII restriction enzymes. The digested DNA fragment was  
ligated into EcoRI/HindIII-digested pKK vector to generate pKK-JUN-HBcAg  
expression vector. Insertion of the PCR product was analyzed by EcoRI/HindIII  
restriction analysis and by DNA sequencing of the insert.

### 15 *Example 18*

#### *Fusion of JUN amphipathic helix to the carboxy terminus of HBcAg(1-144)*

The JUN helix was fused to the carboxy terminus of the HBcAg amino  
acid sequence 1 to 144 (HBcAg-JUN construct). For construction of the HBcAg-  
JUN DNA sequence, the sequences encoding the JUN helix and HBcAg(1-144)  
20 were amplified separately by PCR. The JUN sequence was amplified from the  
pJuFo plasmid with primers SacII-JUN(s) and JUN-HindIII(as). SacII-JUN(s)  
introduced a linker encoding amino acids LAAG. This sequence also contains a  
SacII site. JUN-HindIII(as) introduced a stop codon (TAA) followed by a HindIII  
site. The HBcAg(1-144) DNA sequence was amplified from the pEco63 plasmid  
25 using primers EcoRI-HBcAg(s) and HBcAg(1-144)-JUN(as). EcoRI-HBcAg(s)  
introduced an EcoRI site prior to the Start ATG of the HBcAg coding sequence.  
HBcAg(1-144)-JUN(as) introduces a sequence encoding the peptide linker  
(LAAG), which also contains a SacII site. For the PCR reactions, 100 pmol of

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each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. Temperature cycling was carried out as follows: 94°C for 2 minutes; and 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

5      ***Primer sequences***

SacII-JUN(s):

(5'-CTAGCCGCGGGTTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:65);

JUN-HindIII(as):

10      (5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC -3') (SEQ ID NO:66);

EcoRI-HBcAg(s):

(5'-CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:67);  
and

15      HBcAg-JUN(as):

(5'-CCGACCACCGCAACCCGCGGCTAGCGGAAGCGTTGATAGGATAGG-3')  
(SEQ ID NO:68).

20      Fusion of the two PCR fragments was performed by PCR using primers EcoRI-HBcAg(s) and JUN-HindIII(as). For the PCR fusion, 100 pmol of each oligo was used with 100ng of the purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The final PCR product was analyzed by agarose gel electrophoresis, and digested for 16 hours in an

appropriate buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was gel purified and ligated into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-JUN expression vector. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

### *Example 19*

#### *Insertion of JUN amphipathic helix into the c/e1 epitope of HBcAg(1-144)*

The c/e1 epitope (residues 72 to 88) of HBcAg is known to be located in the tip region on the surface of the hepatitis B virus capsid. A part of this region (residues 76 to 82) of the protein was genetically replaced by the JUN helix to provide an attachment site for antigens (HBcAg-JUNIns construct). The HBcAg-JUNIns DNA sequence was generated by PCRs: The JUN helix sequence and two sequences encoding HBcAg fragments (amino acid residues 1 to 75 and 83 to 144) were amplified separately by PCR. The JUN sequence was amplified from the pJuFo plasmid with primers BamHI-JUN(s) and JUN-SacII(as). BamHI-JUN(s) introduced a linker sequence encoding the peptide sequence GSGGG that also contains a BamHI site. JUN-SacII(as) introduced a sequence encoding the peptide linker GAAGS followed by a sequence complementary to the 3' end of the JUN coding sequence. The HBcAg(1-75) DNA sequence was amplified from the pEco63 plasmid using primers EcoRIHBcAg(s) and HBcAg75-JUN(as). EcoRIHBcAg(s) introduced an EcoRI site followed by a sequence corresponding to the 5' end of the HBcAg sequence. HBcAg75-JUN(as) introduced a linker encoding the peptide GSGGG after amino acid 75 of HBcAg followed by a sequence complementary to the 5' end of the sequence encoding the JUN helix. The HBcAg (83-144) fragment was amplified using primers JUN-HBcAg83(s) and HBcAg(1-144)Hind(as). JUN-HBcAg83(s) contained a sequence corresponding to the 3' end of the JUN-encoding sequence followed by a linker encoding the peptide, GAAGS and a sequence corresponding to the 5' end of the sequence encoding HBcAg (83-144). HBcAg(1-144)Hind(as)

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introduced a stop codon and a HindIII site after codon 144 of the HBcAg gene. For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures (2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>). Temperature cycling was performed as follows:  
5 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

***Primer sequences:***

BamHI-JUN(s):

(5'-CTAATGGATCCGGTGGGGGCTGCGGTGGTCGGATCGCCCGGCTCGAG-3')

10 (SEQ ID NO:69);

JUN-SacII(as):

(5'-GTCGCTACCCGCGGCTCCGCAACCAACGTGGTTCATGAC-3')(SEQ

ID NO:70);

EcoRIHBcAg(s):

15 (5'- CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:71);

HBcAg75-JUN (as):

(5'-CCGACCACCGCAGCCCCACCGGATCCATTAGTACCCACCCAGGTAGC-3')

(SEQ ID NO:72);

JUN-HBcAg83(s):

20 (5'-GTTGGTTGCGGAGCCGCGGGTAGCGACCTAGTAGTCAGTTATGTC-3')

(SEQ ID NO:73); and

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HBcAg(1-144)Hind(as):

(5'-CGCGTCCCAAGCTTCTACGGAAGCGTTGATAGGATAGG-3') (SEQ ID NO:74).

Fusion of the three PCR fragments was performed as follows. First, the  
 5 fragment encoding HBcAg 1-75 was fused with the sequence encoding JUN by  
 PCR using primers EcoRIHBcAg(s) and JUN-SacII(as). Second, the product  
 obtained was fused with the HBcAg(83-144) fragment by PCR using primers  
 EcoRI HBcAg(s) and HBcAg HindIII(as). For PCR fusions, 100 pmol of each  
 10 oligo was used with 100 ng of the purified PCR fragments in a 50 µl reaction  
 mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM  
 MgSO<sub>4</sub>. The same PCR cycles were used as for generation of the individual  
 fragments. The final PCR product was digested for 16 hours in an appropriate  
 buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was  
 15 ligated into EcoRI/HindIII-digested pKK vector, yielding the pKK-HBcAg-  
 JUNIns vector. Insertion of the PCR product was analyzed by EcoRI/HindIII  
 restriction analysis and by DNA sequencing of the insert.

#### *Example 20*

##### *Fusion of the JUN amphipathic helix to the carboxy terminus of the measles virus nucleocapsid (N) protein*

20 The JUN helix was fused to the carboxy terminus of the truncated measles  
 virus N protein fragment comprising amino acid residues 1 to 473 (N473-JUN  
 construct). For construction of the DNA sequence encoding N473-JUN the  
 sequence encoding the JUN helix and the sequence encoding N473-JUN were  
 amplified separately by PCR. The JUN sequence was amplified from the pJuFo  
 25 plasmid with primers SacII-JUN(s) and JUN-HindIII(as). SacII-JUN(s)  
 introduced a sequence encoding peptide linker LAAG. This sequence also  
 contained a SacII site. The JUN-HindIII(as) anti-sense primer introduced a stop  
 codon (TAA) followed by a HindIII site. The N (1-473) sequence was amplified  
 from the pSC-N plasmid containing the complete measles virus N protein coding

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sequence (obtained from M. Billeter, Zurich) using primers EcoRI-Nmea(s) and Nmea-JUN(as). EcoRI-N(meas) introduced an EcoRI site prior to the Start ATG of the N coding sequence. N(meas)-JUN(as) was complementary to the 3' end of the N(1-473) coding sequence followed by a sequence complementary to the coding sequence for the peptide linker (LAAG). For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. Temperature cycling was performed as follows: 94 °C for 2 minutes; and 35 cycles of 94 °C (1 minute), 55 °C (1 minute), 72 °C (2 minutes).

10 ***Primer sequences:***

SacII-JUN(s):

(5'-CTAGCCGCGGGTTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:75);

JUN-HindIII(as):

15 (5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC -3') (SEQ ID NO:76);

EcoRI-Nmea(s):

(5'-CCGGAATTCATGGCCACACTTTTAAGGAGC-3')(SEQ ID NO:77); and

Nmea-JUN(as):

20 (5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC-3')(SEQ ID NO:78).

Fusion of the two PCR fragments was performed in a further PCR using primers EcoRI-Nmea(s) and Nmea-JUN(as). For the PCR fusion, 100 pmol of each oligo was used with 100 ng of the purified PCR fragments in a 50 µl

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reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. Temperature cycling was performed as follows: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The PCR product was digested for 16 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was gel purified and ligated into EcoRI/HindIII-digested pKK vector, yielding the pKK-N473-JUN plasmid. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

### Example 21

#### Expression and partial purification of HBcAg-JUN

*E. coli* strain XL-1 blue was transformed with pKK-HBcAg-JUN. 1 ml of an overnight culture of bacteria was used to inoculate 100 ml of LB medium containing 100 µg/ml ampicillin. This culture was grown for 4 hours at 37°C until an OD at 600 nm of approximately 0.8 was reached. Induction of the synthesis of HBcAg-JUN was performed by addition of IPTG to a final concentration of 1 mM. After induction, bacteria were further shaken at 37°C for 16 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -20°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA, 10 mM DTT) supplemented with 200 µg/ml lysosyme and 10 µl of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted using a French pressure cell. Triton X-100 was added to the lysate to a final concentration of 0.2%, and the lysate was incubated for 30 minutes on ice and shaken occasionally. Figure 4 shows HBcAg-JUN protein expression in *E. coli* upon induction with IPTG. *E. coli* cells harboring pKK-HBcAg-JUN expression plasmid or a control plasmid were used for induction of HBcAg-JUN expression with IPTG. Prior to the addition of IPTG, a sample was removed from the bacteria culture carrying the pKK-HBcAg-JUN plasmid (lane 3) and from a culture carrying the control plasmid (lane 1). Sixteen hours after addition of



IPTG, samples were again removed from the culture containing pKK-HBcAg-JUN (lane 4) and from the control culture (lane 2). Protein expression was monitored by SDS-PAGE followed by Coomassie staining.

5 The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-JUN protein was soluble (Fig. 5).  
10 Briefly, lysates from *E. coli* cells expressing HBcAg-JUN and from control cells were centrifuged at 14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-HBcAg monoclonal antibody YVS 1841. Lane 1: soluble fraction, control cells; lane 2: insoluble fraction, control cells; lane 3:  
15 soluble fraction, cells expressing HBcAg-JUN; lane 4: insoluble fraction, cells expressing HbcAg-JUN.

The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was  
20 centrifuged for 3 hrs with 100,000 x g at 4°C. After centrifugation, 1 ml fractions from the top of the gradient were collected and analyzed by SDS-PAGE followed by Coomassie staining. (Fig. 6). Lane 1: total *E. coli* lysate prior to centrifugation. Lane 1 and 2: fractions 1 and 2 from the top of the gradient. Lane 4 to 7: fractions 5 to 8 (15% sucrose). The HBcAg-JUN protein was  
25 detected by Coomassie staining.

The HBcAg-JUN protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-gradient centrifugation of the HBcAg-JUN particles led both to enrichment and  
30 to a partial purification of the particles.

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### Example 22

#### *Covalent Coupling of hGH-FOS to HBcAg-JUN*

In order to demonstrate binding of a protein to HBcAg-JUN particles, we chose human growth hormone (hGH) fused with its carboxy terminus to the FOS helix as a model protein (hGH-FOS). HBcAg-JUN particles were mixed with partially purified hGH-FOS and incubated for 4 hours at 4°C to allow binding of the proteins. The mixture was then dialyzed overnight against a 3000-fold volume of dialysis buffer (150 mM NaCl, 10 mM Tris-HCl solution, pH 8.0) in order to remove DTT present in both the HBcAg-JUN solution and the hGH-FOS solution and thereby allow covalent coupling of the proteins through the establishment of disulphide bonds. As controls, the HBcAg-JUN and the hGH-FOS solutions were also dialyzed against dialysis buffer. Samples from all three dialyzed protein solutions were analyzed by SDS-PAGE under non-reducing conditions. Coupling of hGH-FOS to HBcAg-JUN was detected in an anti-hGH immunoblot (Fig. 7). hGH-FOS bound to HBcAg-JUN should migrate with an apparent molecular mass of approximately 53 kDa, while unbound hGH-FOS migrates with an apparent molecular mass of 31 kDa. The dialysate was analyzed by SDS-PAGE in the absence of reducing agent (lane 3) and in the presence of reducing agent (lane 2) and detected by Coomassie staining. As a control, hGH-FOS that had not been mixed with capsid particles was also loaded on the gel in the presence of reducing agent (lane 1).

A shift of hGH-FOS to a molecular mass of approximately 53 kDa was observed in the presence of HBcAg-JUN capsid protein, suggesting that efficient binding of hGH-FOS to HBcAg-JUN had taken place.

### Example 23

#### *Insertion of a peptide containing a Lysine residue into the c/e1 epitope of HBcAg(1-149)*

The c/e1 epitope (residues 72 to 88) of HBcAg is located in the tip region on the surface of the hepatitis B virus capsid (HBcAg). A part of this region

(Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (HBcAg-Lys construct). The introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free Cysteine group.

The HBcAg-Lys DNA sequence was generated by PCRs: The two fragments encoding HBcAg fragments (amino acid residues 1 to 78 and 81 to 149) were amplified separately by PCR. The primers used for these PCRs also introduced a DNA sequence encoding the Gly-Gly-Lys-Gly-Gly peptide. The HBcAg (1 to 78) fragment was amplified from pEco63 using primers EcoRIHBcAg(s) and Lys-HBcAg(as). The HBcAg (81 to 149) fragment was amplified from pEco63 using primers Lys-HBcAg(s) and HBcAg(1-149)Hind(as). Primers Lys-HBcAg(as) and Lys-HBcAg(s) introduced complementary DNA sequences at the ends of the two PCR products allowing fusion of the two PCR products in a subsequent assembly PCR. The assembled fragments were amplified by PCR using primers EcoRIHBcAg(s) and HbcAg(1-149)Hind(as).

For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

***Primer sequences:***

EcoRIHBcAg(s):

(5'-CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:79);

Lys-HBcAg(as):

(5'-CCTAGAGCCACCTTTGCCACCATCTTCTAAATTAGTACCCACCCAG

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GTAGC-3') (SEQ ID NO:80);

Lys-HBcAg(s):

(5'-GAAGATGGTGGCAAAGGTGGCTCTAGGGACCTAGTAGTCAGTTAT  
GTC -3') (SEQ ID NO:81);

5 HBcAg(1-149)Hind(as):

(5'-CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAG-3') (SEQ  
ID NO:82).

10 For fusion of the two PCR fragments by PCR 100 pmol of primers  
EcoRIHBcAg(s) and HBcAg(1-149)Hind(as) were used with 100 ng of the two  
purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo  
polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were:  
94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2  
minutes). The assembled PCR product was analyzed by agarose gel  
electrophoresis, purified and digested for 19 hours in an appropriate buffer with  
15 EcoRI and HindIII restriction enzymes. The digested DNA fragment was ligated  
into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-Lys expression  
vector. Insertion of the PCR product into the vector was analyzed by  
EcoRI/HindIII restriction analysis and DNA sequencing of the insert.

#### Example 24

##### 20 *Expression and partial purification of HBcAg-Lys*

*E. coli* strain XL-1 blue was transformed with pKK-HBcAg-Lys. 1 ml of  
an overnight culture of bacteria was used to inoculate 100 ml of LB medium  
containing 100 µg/ml ampicillin. This culture was grown for 4 hours at 37°C  
until an OD at 600 nm of approximately 0.8 was reached. Induction of the  
25 synthesis of HBcAg-Lys was performed by addition of IPTG to a final  
concentration of 1 mM. After induction, bacteria were further shaken at 37°C for

16 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -20°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA, 10 mM DTT) supplemented with 200 µg/ml lysosyme and 10 µl of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted using a French pressure cell. Triton X-100 was added to the lysate to a final concentration of 0.2%, and the lysate was incubated for 30 minutes on ice and shaken occasionally. *E. coli* cells harboring pKK-HBcAg-Lys expression plasmid or a control plasmid were used for induction of HBcAg-Lys expression with IPTG. Prior to the addition of IPTG, a sample was removed from the bacteria culture carrying the pKK-HBcAg-Lys plasmid and from a culture carrying the control plasmid. Sixteen hours after addition of IPTG, samples were again removed from the culture containing pKK-HBcAg-Lys and from the control culture. Protein expression was monitored by SDS-PAGE followed by Coomassie staining.

The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-Lys protein was soluble. Briefly, lysates from *E. coli* cells expressing HBcAg-Lys and from control cells were centrifuged at 14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-HBcAg monoclonal antibody YVS 1841.

The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was centrifuged for 3 hrs with 100,000 x g at 4°C. After centrifugation, 1 ml fractions from the top of the gradient were collected and analyzed by SDS-PAGE followed

by Coomassie staining. The HBcAg-Lys protein was detected by Coomassie staining.

The HBcAg-Lys protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-gradient centrifugation of the HBcAg-Lys particles led both to enrichment and to a partial purification of the particles.

### *Example 25*

#### *Chemical coupling of FLAG peptide to HBcAg-Lys using the heterobifunctional crosslinker SPDP*

Synthetic FLAG peptide with a Cysteine residue at its amino terminus (amino acid sequence CGGDYKDDDDK) was coupled chemically to purified HBcAg-Lys particles in order to elicit an immune response against the FLAG peptide. 600 µl of a 95% pure solution of HBcAg-Lys particles (2 mg/ml) were incubated for 30 minutes at room temperature with the heterobifunctional crosslinker N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (0.5 mM). After completion of the reaction, the mixture was dialysed overnight against 1 liter of 50 mM Phosphate buffer (pH 7.2) with 150 mM NaCl to remove free SPDP. Then 500 µl of derivatized HBcAg-Lys capsid (2 mg/ml) were mixed with 0.1 mM FLAG peptide (containing an amino-terminal cysteine) in the presence of 10 mM EDTA to prevent metal-catalyzed sufhydryl oxidation. The reaction was monitored through the increase of the optical density of the solution at 343 nm due to the release of pyridine-2-thione from SPDP upon reaction with the free cysteine of the peptide. The reaction of derivatised Lys residues with the peptide was complete after approximately 30 minutes.

The FLAG decorated particles were injected into mice.

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*Example 26**Construction of pMPSV-gp140cys*

The gp140 gene was amplified by PCR from pCytTSgp140FOS using oligos gp140CysEcoRI and SalIgp140. For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (0.5 minutes), 55°C (0.5 minutes), 72°C (2 minutes).

The PCR product was purified using QiaEXII kit, digested with SalI/EcoRI and ligated into vector pMPSVHE cleaved with the same enzymes.

*Oligo sequences:*

Gp140CysEcoRI:

5'-GCCGAATTCCTAGCAGCTAGCACCGAATTTATCTAA-3' (SEQ ID NO:83);

SalIgp140

5'-GGTTAAGTCGACATGAGAGTGAAGGAGAAATAT-3' (SEQ ID NO:84).

*Example 27**Expression of pMPSVgp140Cys*

pMPSVgp140Cys (20 µg) was linearized by restriction digestion. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linearized DNA. The restriction digestion was evaluated by agarose gel electrophoresis. For the transfection, 5.4 µg of linearized pMPSVgp140-Cys was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution was added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for

-78-

25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture (6-well plate) was then replaced by the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

Stably transfected cells were selected and grown in selection medium (HP-1 medium supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 h growth period at 37°C. One dish of the cells was shifted to 30°C to induce the expression of soluble GP140-*FOS*. The other dish was kept at 37°C.

The expression of soluble GP140-Cys was determined by Western blot analysis. Culture media (0.5 ml) was methanol/chloroform precipitated, and the pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to a 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-GP140 or GP-160 antibody for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-mouse/rabbit/monkey/human IgG conjugate. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development



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reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50  $\mu$ l NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37  $\mu$ l of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide).

### Example 28

#### Purification of gp140Cys

An anti-gp120 antibody was covalently coupled to a NHS/EDC activated dextran and packed into a chromatography column. The supernatant, containing GP140Cys is loaded onto the column and after sufficient washing, GP140Cys was eluted using 0.1 M HCl. The eluate was directly neutralized during collection using 1 M Tris pH 7.2 in the collection tubes.

Disulfide bond formation might occur during purification, therefore the collected sample is treated with 10 mM DTT in 10 mM Tris pH 7.5 for 2 hours at 25°C.

DTT is remove by subsequent dialysis against 10 mM Mes; 80 mM NaCl pH 6.0. Finally GP140Cys is mixed with alphavirus particles containing the JUN residue in E2 as described in Example 16.

### Example 29

#### Construction of PLA2-Cys

The PLA2 gene was amplified by PCR from pAV3PLAfos using oligos EcoRIPLA and PLA-Cys-hind. For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50  $\mu$ l reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions , temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (0.5 minutes), 55°C (0.5 minutes), 72°C (2 minutes).

The PCR product was purified using QiaEXII kit, digested with EcoRI/HinDIII and ligated into vector pAV3 cleaved with the same enzymes.

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Oligos

EcoRIPLA:

5'-TAACCGAATTCAGGAGGTAAAAAGATATGG-3' (SEQ ID NO:85)

PLACys-hind:

5 5'-GAAGTAAAGCTTTTAACCACCGCAACCACCAGAAG-3' (SEQ ID NO:86).

### Example 30

#### *Expression and purification of PLA-cys*

For cytoplasmic production of Cys tagged proteins, *E. coli* XL-1-Blue strain was transformed with the vectors pAV3::PLA and pPLA-Cys. The culture was incubated in rich medium in the presence of ampicillin at 37°C with shaking. At an optical density (550nm) of , 1 mM IPTG was added and incubation was continued for another 5 hours. The cells were harvested by centrifugation, resuspended in an appropriate buffer (e.g. Tris-HCl, pH 7.2, 150 mM NaCl) containing DNase, RNase and lysozyme, and disrupted by passage through a french pressure cell. After centrifugation (Sorvall RC-5C, SS34 rotor, 15000 rpm, 10 min, 4°C), the pellet was resuspended in 25 ml inclusion body wash buffer (20 mM tris-HCl, 23% sucrose, 0.5% Triton X-100, 1 mM EDTA, pH8) at 4°C and recentrifuged as described above. This procedure was repeated until the supernatant after centrifugation was essentially clear. Inclusion bodies were resuspended in 20 ml solubilization buffer (5.5 M guanidinium hydrochloride, 25 mM tris-HCl, pH 7.5) at room temperature and insoluble material was removed by centrifugation and subsequent passage of the supernatant through a sterile filter (0.45 µm). The protein solution was kept at 4°C for at least 10 hours in the presence of 10 mM EDTA and 100 mM DTT and then dialyzed three times against 10 volumes of 5.5 M guanidinium hydrochloride, 25 mM tris-HCl, 10 mM EDTA, pH 6. The solution was dialyzed twice against 51 2 M urea, 4 mM EDTA, 0.1 M NH<sub>4</sub>Cl, 20 mM sodium borate

-81-

(pH 8.3) in the presence of an appropriate redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine). The refolded protein was then applied to an ion exchange chromatography. The protein was stored in an appropriate buffer with a pH above 7 in the presence of 2-10 mM DTT to keep the cysteine residues in a reduced form. Prior to coupling of the protein with the alphavirus particles, DTT was removed by passage of the protein solution through a Sephadex G-25 gel filtration column.

*What Is Claimed Is:*

1. A composition comprising:

a) a non-naturally occurring molecular scaffold comprising:

(i) a core particle selected from the group consisting

5 of:

(1) a core particle of non-natural origin; and

(2) a core particle of natural origin; and

(ii) an organizer comprising at least one first attachment site,

10 wherein said organizer is connected to said core particle by at least one covalent bond; and

b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

15 (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

20 wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

2. The composition of Claim 1, wherein:

a) said core particle is selected from the group consisting of:

25 (i) a virus

(ii) a virus-like particle;

(iii) a bacteriophage;

(iv) a viral capsid particle; and

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- (v) a recombinant form of (i), (ii), (iii) or (iv); and
- b) said organizer is a polypeptide or residue thereof; and
- c) said second attachment site is a polypeptide or residue thereof.

5                    3.        The composition of Claim 2, wherein said first and/or said second attachment sites comprise:

- a) an antigen and an antibody or antibody fragment thereto;
  - b) biotin and avidin;
  - c) strepavidin and biotin;
  - 10                   d) a receptor and its ligand;
  - e) a ligand-binding protein and its ligand;
  - f) interacting leucine zipper polypeptides;
  - g) an amino group and a chemical group reactive thereto;
  - h) a carboxyl group and a chemical group reactive thereto;
  - 15                   i) a sulfhydryl group and a chemical group reactive thereto;
- or
- j) a combination thereof.

4.        The composition of Claim 3, wherein said second attachment site does not naturally occur with said antigen or antigenic determinant.

20                   5.        The composition of Claim 2, where in said core particle is a recombinant alphavirus.

6.        The composition of Claim 5, wherein said recombinant alphavirus is Sindbis virus and said first attachment site and said second attachment site each comprise an interacting leucine zipper polypeptide.

7. The composition of Claim 6, wherein said first attachment site and said second attachment site are the JUN and/or FOS leucine zipper polypeptides.

8. The composition of Claim 2, wherein said core particle is a virus-like particle.

5 9. The composition of Claim 8, wherein said first attachment site is an amino group and said second attachment site is a sulfhydryl group.

10. The composition of Claim 8, wherein said virus-like particle is a hepatitis B virus capsid protein.

10 11. The composition of Claim 10, wherein said first attachment site and said second attachment site each comprise an interacting leucine zipper polypeptide.

12. The composition of Claim 11, wherein said first attachment site is the JUN polypeptide and said second attachment site is the FOS polypeptide.

15 13. The composition of Claim 10, wherein said first attachment site is a lysine residue and said second attachment site is a cysteine residue.

14. The composition of Claim 8, wherein said virus-like particle is a Measles virus capsid protein.

20 15. The composition of Claim 14, wherein said first attachment site and said second attachment site each comprise an interacting leucine zipper polypeptide.

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16. The composition of Claim 15, wherein said first attachment site and said second attachment site are the JUN and/or FOS leucine zipper polypeptides.

17. The composition of Claim 2, wherein said core particle is selected from the group consisting of:

- a) recombinant proteins of Rotavirus,
- b) recombinant proteins of Norwalk virus,
- c) recombinant proteins of Alphavirus,
- d) recombinant proteins of Foot and Mouth Disease virus,
- e) recombinant proteins of Retrovirus,
- f) recombinant proteins of Hepatitis B virus,
- g) recombinant proteins of Tobacco mosaic virus,
- h) recombinant proteins of Flock House Virus, and
- i) recombinant proteins of human Papillomavirus.

18. The composition of Claim 17, wherein the first attachment site and the second attachment site each comprise an interacting leucine zipper polypeptide.

19. The composition of Claim 17, wherein said first attachment site is an amino group and said second attachment site is sulfhydryl group.

20. The composition of Claim 1, where in said core particle is of non-natural origin.

21. The composition of Claim 20, wherein said core particle is selected from the group consisting of:

- a) synthetic polymer,
- b) a lipid micelle, and

- c) a metal.

22. The composition of Claim 21, wherein said first attachment site and said second attachment site each comprise an interacting leucine zipper polypeptide.

5           23. The composition of Claim 22, wherein said first attachment site and said second attachment site are the JUN and/or FOS leucine zipper polypeptides.

24. The composition of Claim 1, wherein said antigen is selected from the group consisting of:

- 10           a) proteins suited to induce an immune response against cancer cells,
- b) proteins suited to induce an immune response against infectious diseases,
- c) proteins suited to induce an immune response against
- 15           allergens, and
- d) proteins suited to induce an immune response in farm animals.

25. The composition of Claim 24, wherein said antigen is:

- 20           a) a recombinant protein of HIV,
- b) a recombinant protein of Influenza virus,
- c) a recombinant protein of Hepatitis C virus,
- d) a recombinant protein of Toxoplasma,
- e) a recombinant protein of Plasmodium falciparum,
- f) a recombinant protein of Plasmodium vivax,
- 25           g) a recombinant protein of Plasmodium ovale,
- h) a recombinant protein of Plasmodium malariae,



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- 5
- i) a recombinant protein of breast cancer cells,
  - j) a recombinant protein of kidney cancer cells,
  - k) a recombinant protein of prostate cancer cells,
  - l) a recombinant protein of skin cancer cells,
  - m) a recombinant protein of brain cancer cells,
  - n) a recombinant protein of leukemia cells,
  - o) a recombinant profiling,
  - p) a recombinant protein of bee sting allergy,
  - q) a recombinant protein of nut allergy,
  - 10 r) a recombinant protein of food allergies, or
  - s) a recombinant protein of asthma, or
  - t) a recombinant protein of Chlamydia.

15 26. The composition of Claim 24, wherein the first attachment site and the second attachment site each comprise an interacting leucine zipper polypeptide.

27. A process for producing a non-naturally occurring, ordered and repetitive antigen array comprising:

- 20 a) providing a non-naturally occurring molecular scaffold comprising:
- (i) a core particle selected from the group consisting of:
    - (1) a core particle of non-natural origin; and
    - (2) a core particle of natural origin; and
  - (ii) an organizer comprising at least one first
- 25 attachment site,

wherein said organizer is connected to said core particle by at least one covalent bond; and

b) providing an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

(i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and

c) combining said non-naturally occurring molecular scaffold and said antigen or antigenic determinant,

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

28. The process of Claim 27, wherein

a) said core particle is selected from the group consisting of:

(i) a virus

(ii) a virus-like particle;

(iii) a bacteriophage;

(iv) a viral capsid particle; and

(v) a recombinant form of (i), (ii), (iii) or (iv); and

b) said organizer is a polypeptide or residue thereof; and

c) said second attachment site is a polypeptide or residue thereof.

29. The process of Claim 28, wherein said first and/or said second attachment sites comprise:

a) an antigen and an antibody or antibody fragment thereto;

b) biotin and avidin;

c) strepavidin and biotin;

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- d) a receptor and its ligand;
- e) a ligand-binding protein and its ligand;
- f) interacting leucine zipper polypeptides;
- g) an amino group and a chemical group reactive thereto;
- h) a carboxyl group and a chemical group reactive thereto;
- i) a sulfhydryl group and a chemical group reactive thereto;

or

- j) a combination thereof.

30. The process of Claim 29, wherein said second attachment site does not naturally occur with said antigen or antigenic determinant.

31. An isolated recombinant alphavirus comprising in its genome:

- a) a deletion of RNA packaging signal sequences; and
- b) a non-naturally occurring insertion of the *JUN* leucine zipper protein domain nucleic acid sequence in frame with said alphavirus' E2 envelope protein nucleic acid sequence.

32. A host cell comprising the recombinant alphavirus of Claim 31.

33. A method of medical treatment comprising administering to a subject the composition of Claim 1.

34. A pharmaceutical composition comprising:

- a) the composition of Claim 1; and
- b) an acceptable pharmaceutical carrier.

35. A method of immunization comprising administering to a subject a composition comprising:

- a) a non-naturally occurring molecular scaffold comprising:

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(i) a core particle selected from the group consisting of:

- (1) a core particle of non-natural origin; and
- (2) a core particle of natural origin; and

5 (ii) an organizer comprising at least one first attachment site,

wherein at least one said organizer is connected to said core particle by at least one covalent bond; and

10 b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

(i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

15 (ii) an attachment site naturally occurring with said antigen or antigenic determinant,

wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

20 36. The method of Claim 35, wherein said immunization produces an immune response.

37. The method of Claim 35, wherein said immunization produces a humoral immune response.

25 38. The method of Claim 35, wherein said immunization produces a cellular immune response.

39. The method of Claim 35, wherein said immunization produces a humoral immune response and a cellular immune response.

40. The method of Claim 35, wherein said immunization produces a protective response.

5 41. A vaccine composition comprising:

a) a non-naturally occurring molecular scaffold comprising:

(i) a core particle selected from the group consisting

of:

(1) a core particle of non-natural origin; and

10

(2) a core particle of natural origin; and

(ii) an organizer comprising at least one first

attachment site,

wherein at least one said organizer is connected to said core particle by at least one covalent bond; and

15

b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

(i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

20

(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

25

42. The vaccine composition of Claim 41 further comprising an adjuvant.

43. The vaccine composition of Claim 41, wherein:

- a) said core particle is selected from the group consisting of:
  - (i) a virus
  - (ii) a virus-like particle;
  - (iii) a bacteriophage;
  - (iv) a viral capsid particle; and
  - (v) a recombinant form of (i), (ii), (iii) or (iv); and
- b) said organizer is a polypeptide or residue thereof; and
- c) said second attachment site is a polypeptide or residue

thereof.

44. The vaccine composition of Claim 43, wherein said first and/or said second attachment sites comprise:

- a) an antigen and an antibody or antibody fragment thereto;
- b) biotin and avidin;
- c) strepavidin and biotin;
- d) a receptor and its ligand;
- e) a ligand-binding protein and its ligand;
- f) interacting leucine zipper polypeptides;
- g) an amino group and a chemical group reactive thereto;
- h) a carboxyl group and a chemical group reactive thereto;
- i) a sulfhydryl group and a chemical group reactive thereto;

or

- j) a combination thereof.

45. The vaccine composition of Claim 43, wherein said core particle comprises a virus-like particle.

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46. The vaccine composition of Claim 45, wherein said core particle comprises a Hepatitis B virus-like particle.

47. The vaccine composition of Claim 45, wherein said core particle comprises a measles virus-like particle.

5           48. The vaccine composition of Claim 43, wherein said core particle comprises a virus.

49. The vaccine composition of Claim 48, wherein said core particle comprises the Sindbis virus.

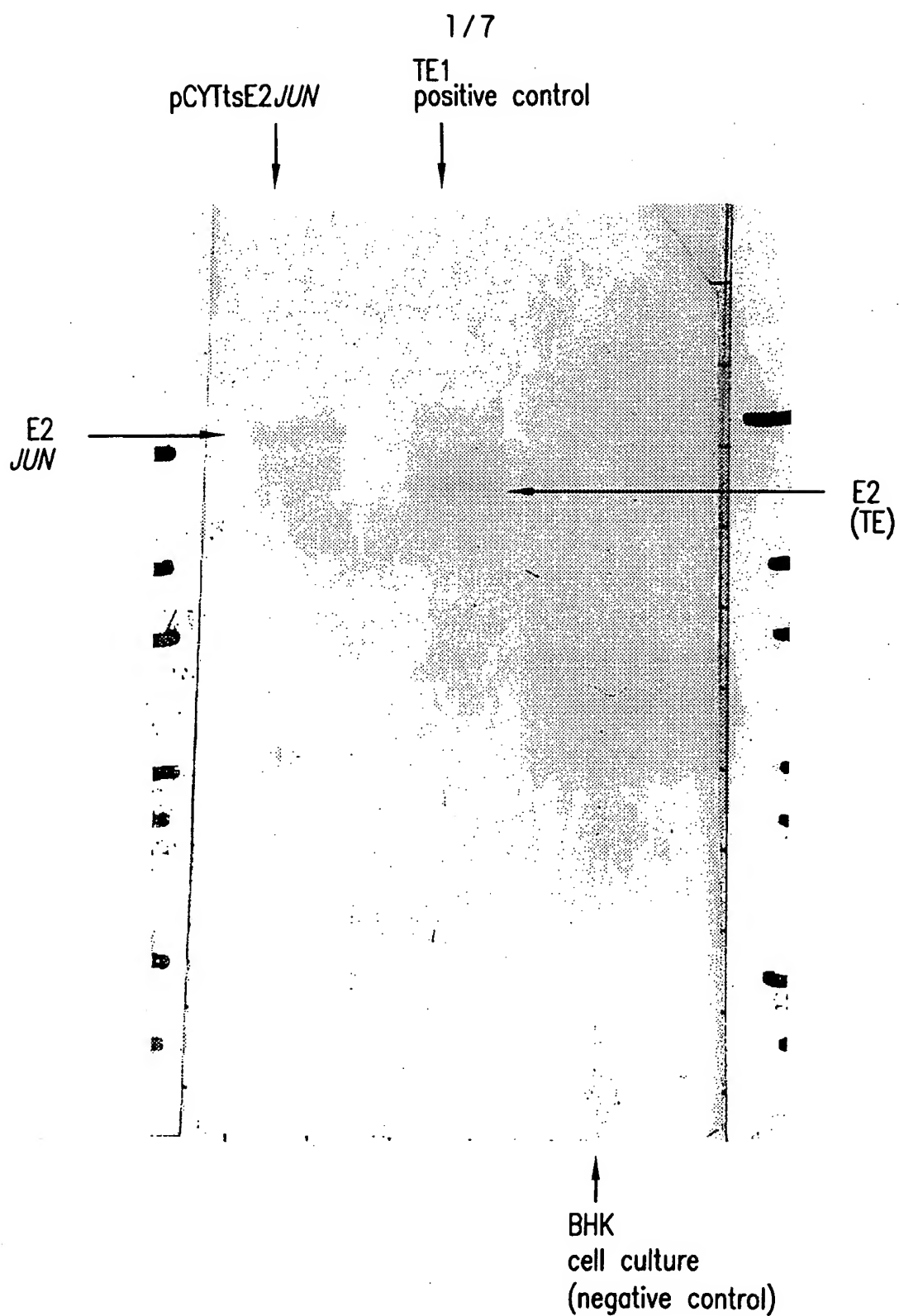


FIG. 1



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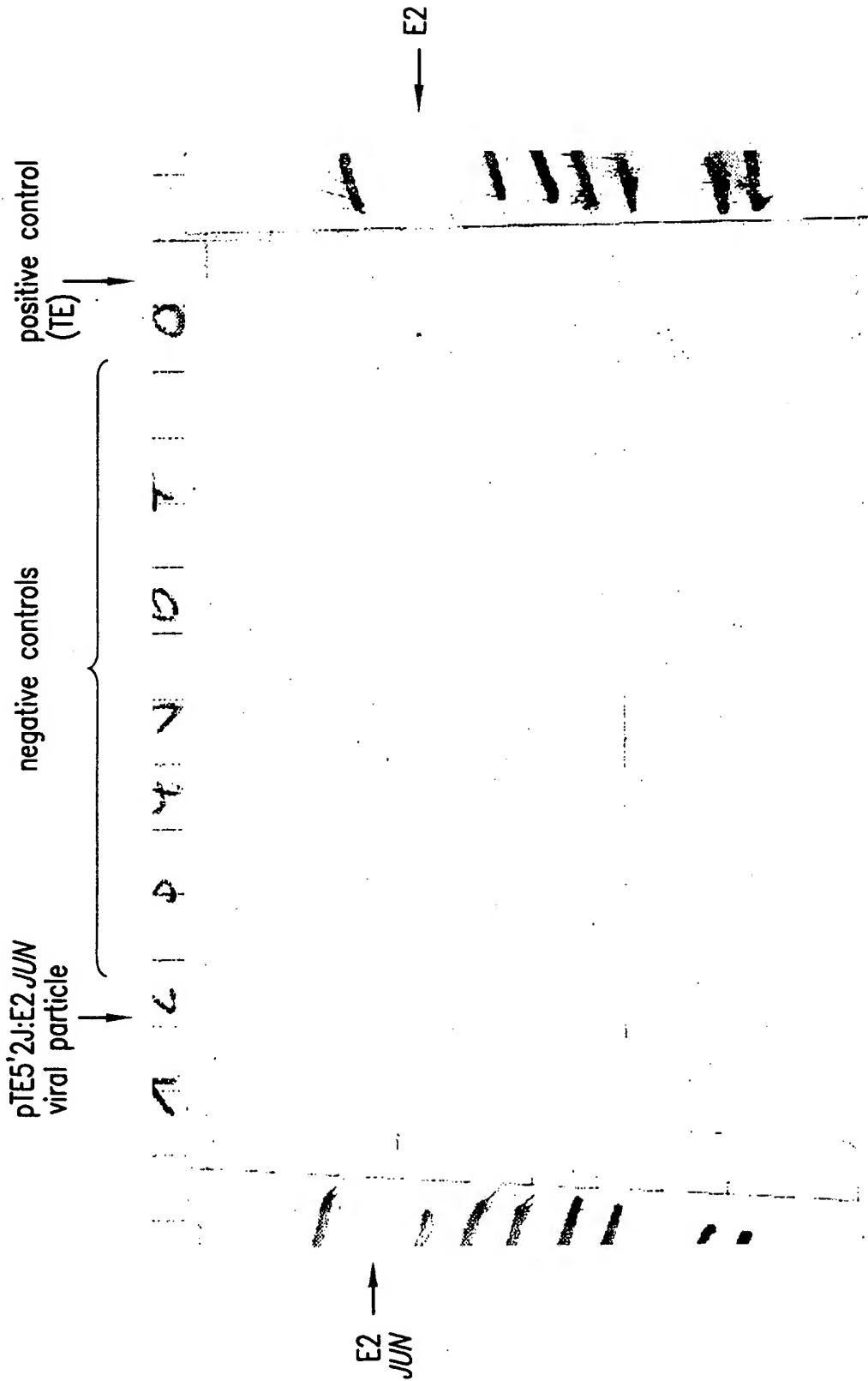


FIG.2

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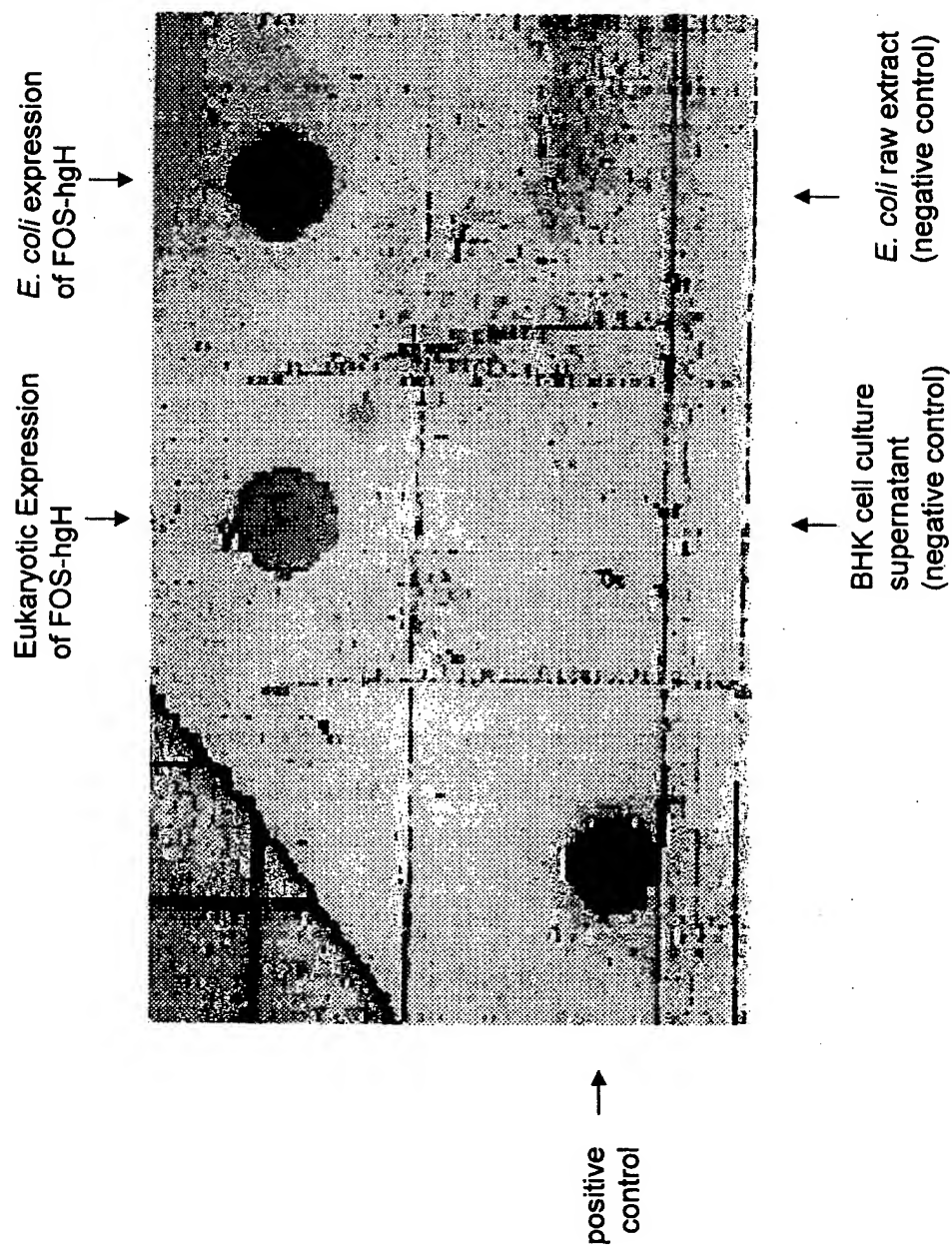


FIG.3

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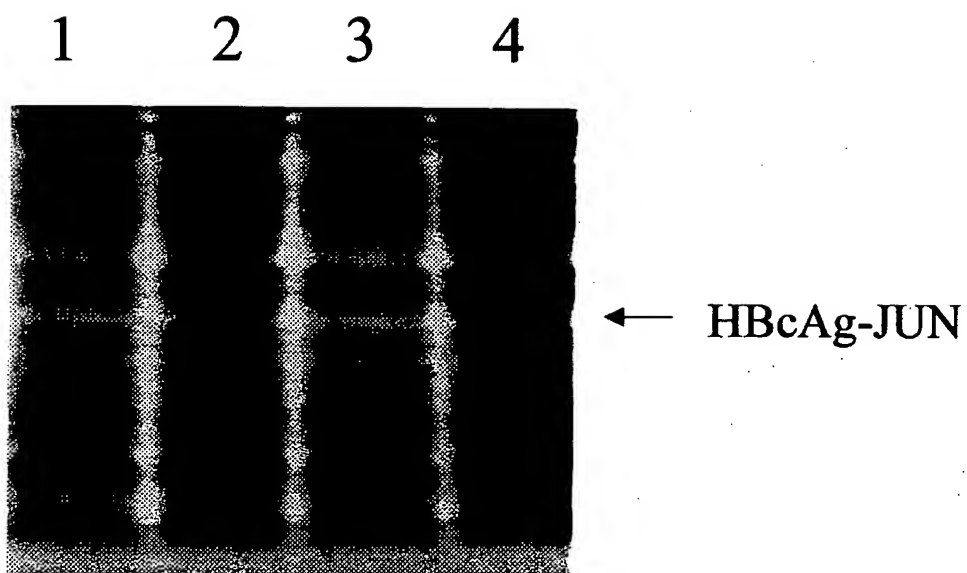


FIG.4

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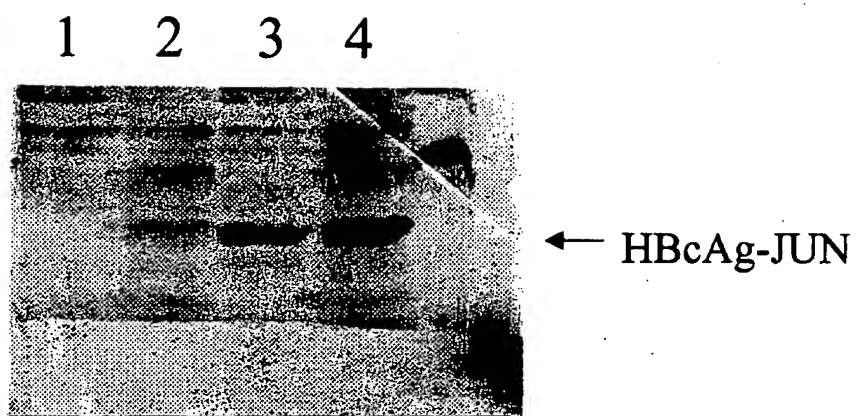


FIG.5

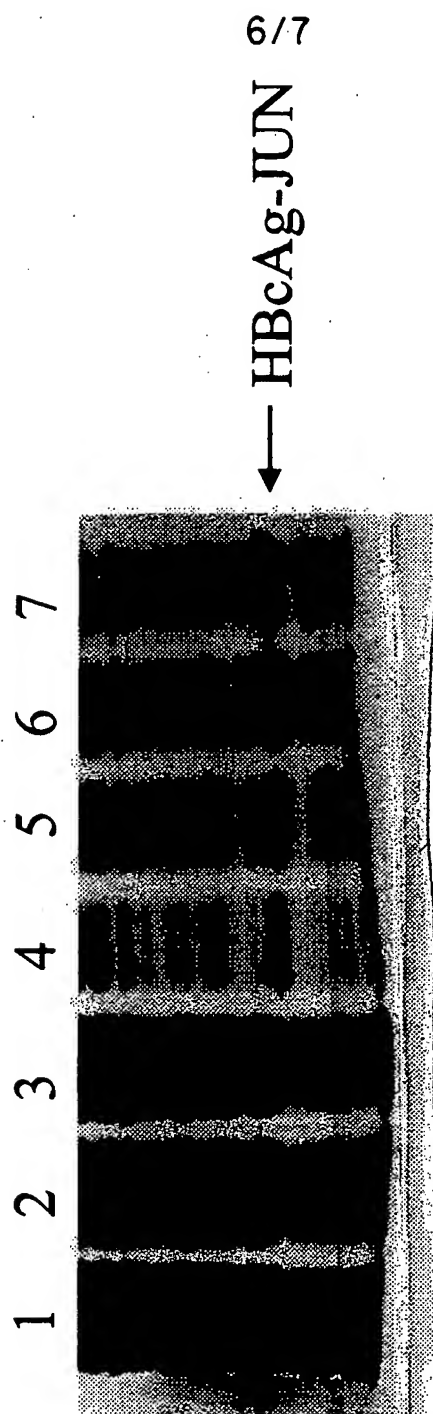


FIG. 6

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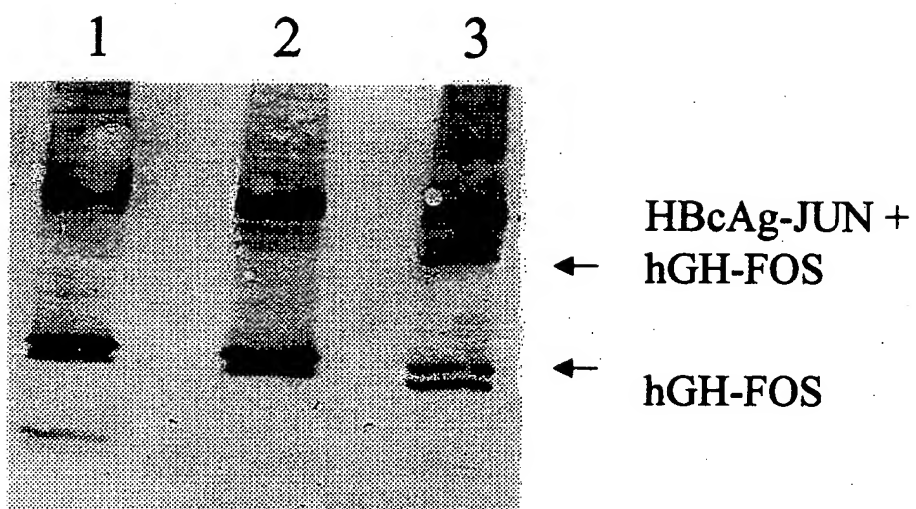


FIG.7

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